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Human Lymphocyte Subpopulations

Studied by Surface Markers and Cytotoxic Activities in Vitro

TORGNY HALLBERG

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HUMAN LYMPHOCYTE SUBPOPULATIONS

Studied by Surface Markers and Cytotoxic Activities in Vitro

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This thesis is based on the following publications:

- I Hallberg, T., Gurner, B.W. and Coombs, R.R.A. (1973) Opsonic adherence of sensitized ox red cells to human lymphocytes as measured by rosette formation. *Int. Arch. Allergy* 44, 500-513.
- II Hallberg, T., Haegert, D., Klein, G.P., Coombs, R.R.A., Feinstein, A. and Gurner, B.W. (1974) Observations on the mixed antiglobulin reaction as a test for immunoglobulin-bearing lymphocytes in normal persons and in patients with chronic lymphatic leukaemia. *J. Immunol. Methods* 4, 317-332.
- III Haegert, D.G., Hallberg, T. and Coombs, R.R.A. (1974) B and T lymphocyte subpopulations in human peripheral blood. *Int. Arch. Allergy* 46, 525-538.
- IV Hallberg, T. (1974) Inhibition of cytotoxicity of non-immune human lymphocytes for sensitized chicken erythrocytes by aggregated human IgG. *Scand. J. Immunol.* 3, 117-125.
- V Hallberg, T. (1974) In vitro cytotoxicity of human lymphocytes: Reduction of the lymphocyte cytotoxicity induced by antibodies or phytohemagglutinin by removing immune-complex-binding cells. *Scand. J. Immunol.* 3, 645-654.

In the text these papers will be referred to by their Roman numerals given above.

GLOSSARY

The following abbreviations are frequently used in the text:

CLL	Chronic lymphatic leukaemia
CRL	Complement receptor lymphocyte
IC	Immune complex
Ig	Immunoglobulin
MAR	Mixed antiglobulin reaction
PHA	Phytohaemagglutinin
RBC	Red blood cell

I. INTRODUCTION

An extensive amount of evidence has established the lymphocyte as the "key cell" of the immune system. Initially considered as the progenitor of effector cells in homograft immunity and in delayed type reactions such as the tuberculin reaction or contact dermatitis, lymphocytes have by now shown a broad diversity of functions with bearing upon both humoral and cell mediated immunity (For review see i.a. WHO technical report 1970, Davies and Carter 1972, Greaves et al 1973).

The discovery by Miller (1961) of the functional importance of the thymus for the maturation of stem cells to a distinct population of lymphocytes, now called thymus-dependent or T lymphocytes, and the analogous observation by Warner and co-workers in 1962 (see Warner and Szenberg, 1964) in the chicken of a bursa-dependent lymphocyte population, now called B lymphocytes or thymus-independent lymphocytes, established functionally heterogenous populations among morphologically indistinguishable lymphocytes.

In the classical concept T and B lymphocytes were considered as progenitors of effector cells in cell-mediated and humoral immunity, respectively. A more dynamic aspect was imposed when T lymphocytes were shown to be essential for the secondary humoral immune response to certain antigens (reviewed in Claman and Chaperon 1969, Claman and Mosier 1972), the T lymphocytes acting as "helper cells" for the antibody-producing cells of B origin. It is still not known if these helper cells are derived from the same T cell population as are the effector cells ("killer cells") in T cell mediated cellular immune reactions.

Activated T cells may produce a variety of so called "lymphokines" (see David and David 1972, WHO technical report 1973), with effector functions (macrophage activation and migration inhibition, cytotoxicity etc.) upon surrounding cells. It is not known if these lymphokines are all produced by the same or different populations of T lymphocytes, and it has not been excluded that some of these factors may be produced by B lymphocytes as well.

Recently it has become evident that the B lymphocyte population, which because of the lack of an identified bursa-analogue in mammals more appropriately may be called non-T lymphocytes, might show functions other than differentiation into antibody-secreting plasma cells. Thus, the cytotoxicity of non-immune lymphocytes for antibody-coated target cells is a property of non-T cells (see Perlmann et al 1972 b). Likewise, lymphocyte cytotoxicity induced by certain mitogens is clearly dependent upon non-T cells as discussed in section VI:C of this thesis. Further, effector cells of non-T origin are operative in cytotoxicity reactions at certain stages of some neoplastic diseases (Lamon et al 1972, O'Toole et al 1973). It is not known whether the "non-T killer cells" operating in these *in vitro* systems are all identical or not.

Most of the data referred to above have been collected in animal experiments, especially inbred mice. They show that for an understanding of lymphocyte functions in healthy and diseased humans, both morphological markers and functional criteria for possible lymphocyte subpopulations are needed. The present thesis is focused on the use of surface markers for the identification of lymphocyte subpopulations in healthy humans and in patients with chronic lymphatic leukaemia, as

well as on finding out which are the effector cells in certain cytotoxicity reactions performed by non-immune human lymphocytes.

II. AIM OF THE INVESTIGATION

Three main questions were asked in the present investigation:

1. How are 4 cell surface markers (*i.e.* receptors for immunoglobulin, receptors for complement, surface immunoglobulin determinants and receptors for sheep red cells) distributed among peripheral blood lymphocytes in healthy humans as compared to patients with chronic lymphatic leukaemia? (I, II, III). Do individual lymphocytes bear more than one of these markers? (I, III).
2. Does polyclonal human IgG inhibit the *in vitro* cytotoxicity of non-immune human lymphocytes for target cells coated with rabbit antibodies? (IV). If so, is the inhibitory activity of IgG dependent upon IgG aggregates, interacting with the effector lymphocytes? (IV).
3. Are lymphocytes bearing receptors for immunoglobulin needed in lymphocyte cytotoxicity reactions induced by target cell antibodies or by phytohaemagglutinin? (V).

During the investigation three main technical points were elaborated:

First, the development of a technique by which binding of immunoglobulin to the surface of human lymphocytes could be easily shown, e.g. by rosette-formation with a suitable indicator cell (I).

Second, the modification of the mixed antiglobulin reaction for detection of Ig-bearing lymphocytes in such a way as to reach a sensitivity on a level with that of immunofluorescence techniques (II).

Third, the application of the rosette-formation techniques for fractionation of different lymphocyte populations (V).

III. MATERIALS

A. Lymphocyte Donors

Lymphocytes were obtained from the following categories:

1. *Healthy individuals* consisting of medical students or laboratory personnel of either sex (I-V).
2. *Patients with chronic lymphatic leukaemia* (I-III).

In addition, 2 patients diagnosed as lymphosarcoma were included in the clinical material investigated in paper II. Diagnostic criteria are given in papers II and III.

IV. METHODS

A. Detection of Cell Surface Markers on Human Lymphocytes

This was performed by the binding to the lymphocyte cell surface of a suitable indicator cell in a so-called rosetting reaction (For review of rosetting techniques see Coombs and Franks 1969). The following surface markers have been studied:

1. *Receptors for antigen-complexed immunoglobulin, so-called Fc-receptors* as shown by the binding of ox red cells sensitized with rabbit antibodies (Figure 1).
2. *Surface immunoglobulin* as shown in the mixed antiglobulin reaction (Figure 2).
3. *Receptors for complement, so-called C3-receptors* as shown by the binding of ox red cells sensitized with rabbit IgM antibodies and human complement (Figure 3).
4. *Receptors for sheep red cells* using non-sensitized sheep red cells as the indicator cells (Figure 4).

The procedures for performing these tests have been described in detail in the individual papers (I–III, V).

B. Lymphocyte Cytotoxicity Experiments

These were performed according to two different models (for review of lymphocyte cytotoxicity assays see Perlmann and Holm 1969):

1. *Antibody-induced lymphocyte cytotoxicity*

In this system purified peripheral blood lymphocytes from healthy humans were activated to cytotoxicity by small amounts of rabbit antibodies sensitizing the target cells.

2. *PHA-induced lymphocyte cytotoxicity*

In this system purified peripheral blood lymphocytes from healthy humans were activated to cytotoxicity for the target cells by including PHA in the cultivation medium.

In both systems erythrocytes from young chickens were used as the target cells and the cytotoxicity was evaluated by the release of ⁵¹chromium from the target cells. The methods have originally been described by Perlmann and Perlmann (1970) and technical details are given in papers IV and V, and in Hallberg (1972).

V. HUMAN LYMPHOCYTE SURFACE MARKERS

A. Receptors for Immune Complexes

1. *Basic data on the binding of immune complexes to non-lymphoid cells*

Immunoglobulins forming complexes with antigens may bind to the surface of certain nucleated cells. Thus, antibody-sensitized particles (*e.g.* bacteria, erythrocytes) bind to phagocytic cells such as macrophages (Berken and Benacerraf 1966),

monocytes (Huber and Fudenberg 1968, Henson 1969) and granulocytes (Henson 1969, Messner and Jelinek 1970) via the Fc-part of the sensitizing antibody (Berken and Benacerraf 1966). This adherence has been referred to as opsonic adherence (Tizard 1970) and is considered to be an important step in the phagocytic process. Various aspects of the phenomenon of opsonic adherence have been reviewed by Coombs and Franks (1969). Aside from phagocytes certain tumour cells also show strong opsonic adherence for antibody-sensitized erythrocytes (Cohen et al 1971, Tönder and Thunold 1973).

The opsonic adherence reaction shows a restriction in the species constellation of sensitizing antibodies and phagocytic cells. Thus, for example, rabbit neutrophils bind red cells sensitized with rabbit but not guinea pig antibodies whereas the contrary is true for guinea pig neutrophils; human neutrophils on the other hand bind red cells sensitized with either of these immunoglobulins (Henson 1969). The almost exclusive binding of IgE to basophils and mast cells from the homologous species also exemplifies this restriction (see Ishizaka 1970, Spiegelberg 1974).

In addition, it is evident that only antibodies of certain classes and subclasses of Ig participate in opsonic adherence phenomena. This was first shown in the guinea pig, in which $\gamma 2G$ but not $\gamma 1G$ can bind opsonically to macrophages (Berken and Benacerraf 1966). Investigations by Huber and Fudenberg (1968) and by Messner and Jelinek (1970) showed binding of human IgG1 and IgG3 but not IgG2 or IgG4 to human monocytes and neutrophils. However, studies using more sensitive techniques have in addition provided evidence for binding of human IgG2 and IgG4 to monocytes and neutrophils and IgA to neutrophils (Henson et al 1972, Spiegelberg 1974). It thus seems as if the capacity of Ig to bind to cell surfaces is, within certain limits, not an all-or-none phenomenon but is related to the degree of complementarity between the receptor on the cell surface and critical structures in the Fc-part of the Ig molecule. It is not known, however, whether a single receptor or a set of different receptors accounts for the binding of the various Ig classes and subclasses. Further data and references on the binding of Ig to phagocytic cells can be found in Unanue (1973) and Spiegelberg (1974).

There is some disagreement as to whether or not the phenomenon of opsonic adherence (i.e. binding of antibody-sensitized particles) is basically identical to that of binding of free cytophilic antibodies (Boyden and Sorkin 1960) to cell surface receptors (for discussion see Coombs and Franks 1969, p. 228). It is clear that cytophilic antibodies can also be demonstrated in opsonic adherence reactions. On the other hand, certain opsonic adherence reactions are not easily reproduced when tested in a cytophilic antibody system. For example, non-complexed IgG binds very inefficiently to lymphocytes in a cytophilic test system, whereas receptors for Ig are easily shown in an opsonic rosetting test (see below section V:A:2). Two explanations could be offered why the opsonic adherence reaction is sometimes positive, although cytophilic antibodies cannot be demonstrated:

1. Binding of antibody to antigen leads to conformational changes exposing in the antibody molecule "new" structures matching to distinct receptors on the cell surface.

2. Binding of antibodies to a multiple-determinant antigen leads to the formation of multiple binding sites thus increasing the total binding force between the antigen-antibody-complex and the phagocytic cell.

Most data, for example the experiments performed by Phillips-Quagliata et al (1971) seem to be in favour of the "multiple-determinant" - hypothesis (see also discussion by Metzger 1974). However, the idea favoured by Coombs and Franks (1969) that in certain systems opsonizing antibodies may expose new binding sites upon combination with antigen has not yet been excluded.

2. *Binding of immune complexes to lymphocytes (I, III)*

Basten et al (1972 a, b) demonstrated that lymphocytes might also possess receptors for immune complexes as first indicated by Uhr (1965) in the guinea pig and LoBuglio et al (1967) in man. That such receptors might exist was also anticipated by the experiments by Bloch-Shtacher et al (1968) and Möller (1969) who found that immune complexes had a mitogenic effect on lymphocytes *in vitro*. Furthermore, an important discovery was that non-immune lymphocytes are cytotoxic for immune-complex-bearing target cells (cf. Perlmann and Holm 1969 and section VI of this thesis).

When the present investigation (I) was begun no simple technique for the demonstration of a possible population of immune complex-binding lymphocytes was available. In preliminary experiments it was found that ox red cells sensitized with rabbit antibodies would bind to a population of human peripheral lymphocytes and an investigation was started to further characterize this reaction and the receptor-bearing lymphocytes (I). In the text below this *receptor* for immunoglobulin will be termed immune-complex (IC) receptor or Fc-receptor to avoid confusion with membrane associated immunoglobulin *determinants*.

It was reported (I) that in 7 healthy individuals 24 to 59 per cent (mean 38,6 %) of peripheral blood lymphocytes carried the IC-receptor. Higher values (41–87 %) were seen in 7 patients with chronic lymphatic leukaemia (CLL). The results in the CLL group will be commented upon below. In a later investigation (III) of 20 healthy individuals a mean of 26.3 % (range 13–50 %) of IC-binding lymphocytes was found.

In the mouse Basten et al (1972 a, b) demonstrated lymphocyte binding of antigen-complexed IgG antibodies by radioautography and Dickler and Kunkel (1972) have shown binding of fluorescein labelled aggregated human IgG to about 22 per cent of peripheral lymphocytes from healthy humans. In contrast, non-aggregated Ig only weakly bound to lymphocyte cell membranes. Brain and Marston (1973) found that human red cells sensitized with incomplete Rh antibodies would also bind to certain human lymphocytes (18–35 % reacting lymphocytes in normal donors). Fröland and Natvig (1973), using the technique of Brain and Marston, found 4.3–39.2 % (mean 14.5 %) rosette-forming lymphocytes in 100 normal adults, whereas somewhat lower values were seen when using indicator cells sensitized with rabbit IgG antibodies. Other investigators, for example Jondal et al (1973), have

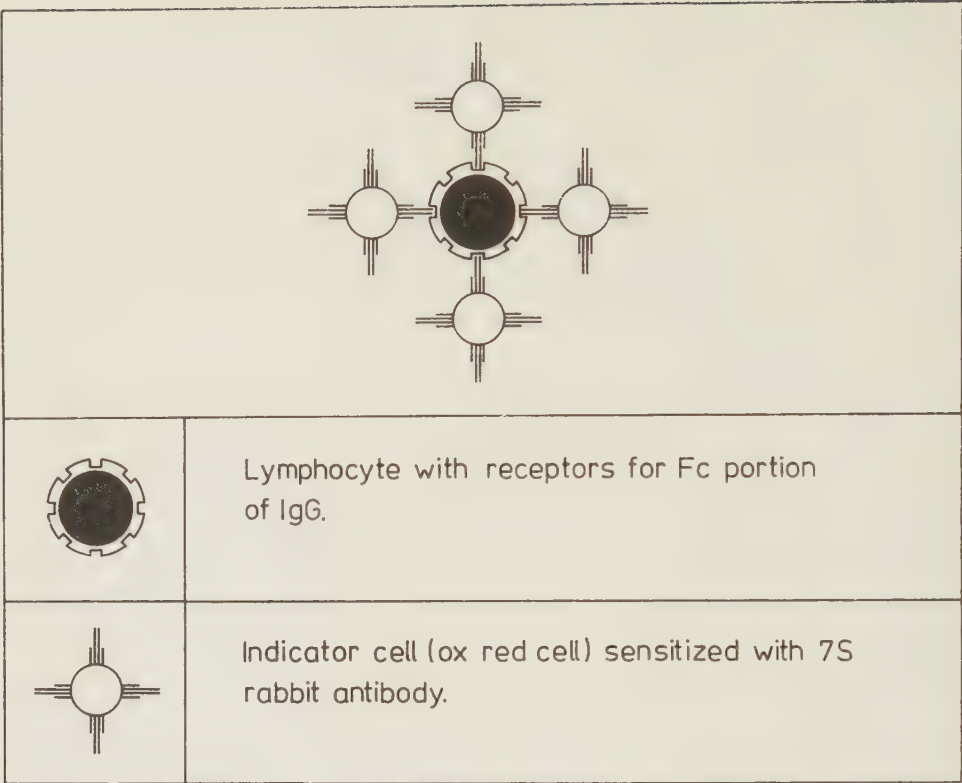


Figure 1. Detection of Fc-receptors on lymphocytes.

had difficulties in demonstrating Fc-binding lymphocytes by anti-Rh-sensitized human erythrocytes. In our experience the results of rosetting tests are much dependent upon the indicator cells used and ox cells seem to be well suited for this purpose. There are, however, qualitative differences between different ox red cells since the agglutinability of the red cells vary between individual oxen (Gleeson-White et al 1950), probably due to the amount of glycoproteins on the cell surface (Uhlenbruck et al 1967), being high in cells of the inagglutinable class. We have found that in Fc-rosetting experiments, ox cells of the most agglutinable class form the strongest rosettes, and thus are to be preferred in the test system.

It was observed that the results in the opsonic adherence test were highly dependent upon the amount of antibody on the indicator ox red cells (I). In this first investigation it could not be ascertained whether there was also a qualitative difference between various antisera. In a later investigation (III), it was shown that the sensitizing antibodies appeared in the 7S peak on Sephadex G-200 filtration, whereas the 19S fraction was completely inactive, in spite of a high agglutinating activity. This indicated that rabbit 7S Ig rather than IgM contained structures matching with receptors on human lymphocytes. Similar conclusions have been drawn from cytotoxicity experiments showing that 7S antibodies, but not 19S antibodies, can induce

lymphocyte cytotoxicity for sensitized target cells (Perlmann and Perlmann 1970). Basten et al (1972 b) showed efficient binding of mouse IgG1 to mouse lymphocytes whereas IgG2b and IgM bound only weakly.

Recent studies have shown that human lymphocytes probably bind all 4 subclasses of human IgG (with the possible exception of IgG4) but do not bind IgM (for references see Spiegelberg 1974). Thus, there is a heterogeneity in the binding affinity of various Ig classes and possibly also subclasses for receptors on lymphocytes. To what extent heterologous immunoglobulins are bound to human lymphocytes is not well known, but obviously rabbit antibodies function well in both opsonic adherence and cytotoxicity tests using human lymphocytes.

Data on the submolecular specificity of the IC receptor (or receptors) are so far sparse. It is well established that the Fc-part of the heavy chain contains the binding site of reacting Ig:s (Basten et al 1972 b, see also Warner (1974) and section VI of this thesis). Thus, the term "Fc-receptor" was coined and it has been used in the present investigation as well. Whether the reacting site is situated in the CH2 or CH3 regions of the heavy chain is a matter of controversy (Wisloff et al 1974, MacLennan 1972 a, MacLennan et al 1974). Neither is it known whether a single lymphocyte carries one or several receptors specific for Ig of various subclasses and of different species origin. It should be mentioned that very recently Fröland et al (1974) have argued that receptors for aggregated Ig, in contrast to receptors for IgG-sensitized erythrocytes ("Fc-receptors") may bind the Fab-part of IgG. Also, in their investigation, receptors for aggregated IgG and Fc-receptors seemed to occur on different lymphocyte populations. In contrast, Dickler (1974) has provided evidence that in his system binding of aggregated Ig is dependent upon an intact Fc-fragment of IgG.

The opsonic adherence of sensitized ox red cells to human lymphocytes was little dependent upon the temperature (+4 to +37°C) (I) and similar observations were made by Dickler and Kunkel (1972) for the binding of aggregated IgG. Routinely, the test was performed at +4°C.

Lymphocytes stored in phosphate-buffered saline at +4°C gradually lost their ability to form rosettes with sensitized ox red cells (I). Likewise, killing of the lymphocytes by freezing, fixation in formaldehyde or treatment with sodium azide extinguished the reactivity. This was in contrast to the increased reactivity of lymphocytes in the mixed antiglobulin reaction for detection of membrane-associated Ig after fixation of the lymphocytes in formaldehyde. The interpretation of this finding is not clear at the moment. It could mean that the Fc-receptor is lost when the cell dies, or that receptor recombinations or other reactions secondary to the primary receptor-determinant binding are essential steps in the rosette-formation procedure between the sensitized indicator cell and the lymphocyte.

The receptor for Fc of IgG acts similar to an anti-immunoglobulin and the question thus arises whether it is by itself of immunoglobulin nature. Indeed, it has been shown that in exceptional cases the surface IgM of CLL lymphocytes has anti-Ig activity (Preud'homme and Seligmann 1972 a). Pretreating the lymphocytes with rabbit anti-human IgM (H+L chains) serum reduced the rosette-formation from 48 % to 15 % rosettes (I). A more doubtful inhibition was seen in the

presence of anti-human Fab serum, whereas normal rabbit or sheep sera or anti-ovalbumin serum had no influence.

Basten et al (1972 b) and Dickler and Kunkel (1972) were unable to block the binding of IC or aggregated Ig to lymphocytes by pretreating the lymphocytes with anti-Ig serum. The reason for the discrepancies between these and our investigations is unclear but may be related to the antisera used and the particular technique for detecting Fc-receptors. However, inhibitory effects of anti-immunoglobulin sera cannot be taken as definite proof that the Fc-receptors are of immunoglobulin nature. That the inhibition was due to soluble IC present in the antiserum seems unlikely, since the serum was not absorbed to make it class-specific. Still, the possibility remains that such complexes could form with IgM released from the lymphocytes. Steric hindrance phenomena, the anti-Ig antibodies binding to structures closely related to the Fc-receptor, could also explain the observed inhibition. In the mouse, Ig determinants and Fc-receptors are indeed closely associated (Basten et al 1972 b).

It should also be recalled that other cells such as phagocytes and tumour cells presumably lacking the capacity to synthesize immunoglobulins, may bind immune complexes as mentioned above. Also, the Fc-part of certain immunoglobulins have structures which bind to well-defined non-Ig proteins such as C1q and protein A of *Staphylococcus aureus* (for references see Spiegelberg 1974).

Evidence that the Fc-receptor is not identical with the complement receptor on mouse lymphocytes was given by Eden et al (1973 a), although a considerable population of lymphocytes carried both receptors.

Soluble antigen-antibody complexes, prepared in the antigen excess region, inhibited the opsonic adherence of sensitized ox red cells to human lymphocytes (I). This was probably due to a competitive blocking of the receptor on the lymphocyte. In analogy, the cytotoxic activity of normal human lymphocytes for antibody-coated chicken erythrocytes was inhibited in the presence of small aggregates of human IgG (see IV and section VI:B of this thesis). Pretreating lymphocytes with normal serum, and then washing the cells, did not inhibit the rosette-formation with sensitized ox red cells (I). However, when lymphocytes were incubated with normal serum and then used in rosetting experiments without a prior wash, a pronounced inhibitory effect was seen (I). It is supposed that this was due to blocking by binding of Ig in the serum to IC receptors on the lymphocytes. It was of interest that heating at $+63^{\circ}\text{C}$ did not increase the blocking activity of the serum (I) since accordingly, heat aggregation did not increase the blocking capacity of human IgG in antibody-induced lymphocyte cytotoxicity (IV). The binding of non-complexed Ig to the lymphocytes is rather loose (Basten et al 1972 a) which may explain why the inhibitory activity was not seen after washing the cells.

Evidence has earlier appeared that the binding of sheep erythrocytes to certain human lymphocytes is a property of T cells (see section V:D). To test whether the Fc-rosetting lymphocytes also would bind sheep erythrocytes fresh lymphocytes were centrifuged with a mixture of sensitized ox RBC and non-sensitized, fluorescein-conjugated sheep RBC (I, III). It was found that the vast majority of Fc- and sheep RBC rosettes formed on different cells and among 4 normal donors (III) at

most 4 % of the rosettes were mixed. Earlier (I) it was reported that 21 % and 8 %, respectively, of mixed rosettes occurred in 2 healthy donors. The higher figures of mixed rosettes obtained in paper I are aberrant from the results in other investigations indicating that T cells generally do not carry Fc-receptors (see Transplant. Rev. 1973, vol. 16) and might be due to non-specific adherence phenomena. However, it is known that Fc-receptors may occur on activated T cells in the mouse (Yoshida and Anderson 1972, van Boxel and Roentreich 1974) and with sensitive techniques 70–85 % of mouse spleen cells and 20–45 % of thymus cells are found to bind aggregated mouse IgG (Anderson and Grey 1974). Thus it seems to be of interest to further analyze the distribution of Fc-receptors on various human lymphocyte populations (see also discussion by Shevac et al 1973).

3. *Fc-receptors on lymphocytes from patients with chronic lymphatic leukaemia*

In paper I it was reported that lymphocytes from a majority of CLL patients had an increased percentage of Fc-receptor lymphocytes, and in none of 7 patients was a subnormal value seen. This was further confirmed in paper III in which 11 CLL patients had a mean of 60 % (range 30–87 %) compared with a mean of 26 % rosettes in healthy individuals. The percentage of rosetting lymphocytes did not correlate with peripheral lymphocyte counts in the patients. In a majority of patients there was a dominance of Ig-bearing lymphocytes over Fc-receptor lymphocytes. In some patients (for example Fox and Ave.) this discrepancy was quite impressive. The opposite situation, i.e. an overwhelming majority of Fc-rosetting lymphocytes over Ig-bearing lymphocytes, was not seen. In all patients the sheep RBC-rosettes were low, consistent with the notion that the leukaemic cells were of B cell origin. In contrast to our results Dickler et al (1973) found binding of aggregated human IgG to be a more sensitive B cell marker in CLL than detection of surface Ig by immunofluorescence. The discrepancies in the techniques used in their and in our investigations could among other things account for these differences (see also Fröland et al 1974). Shevac et al (1972) using IgG-sensitized sheep RBC were unable to find any Fc-binding cells in 6 patients with CLL. However, their technique was intended for the detection of Ig receptors on monocytes and was probably inadequate for the demonstration of lymphocyte Fc-receptors. Selgmann et al (1973) have described a CLL patient with negative surface Ig staining but bearing receptors for aggregated IgG on the lymphocytes.

The results in the C3 rosetting reaction will be commented upon in more detail in section V:C:3. Here it is enough to say that although the mean percentages of the Fc and C3 tests were very similar in the CLL group (60 % and 56 % respectively) remarkable differences were seen in individual patients (for example Smi with 72/21 per cent and Ave with 44/97 per cent of Fc/C3 rosettes (III). This gave further evidence that these receptors occurred as independent entities on leukaemic lymphocytes (see also Brown et al 1974).

In the normal individuals we generally found more Ig-bearing than Fc-rosetting lymphocytes, although the opposite situation was seen in a few individuals (III). Thus the strict correlation between Ig-bearing and IC-binding lymphocytes repre-

ed by Dickler and Kunkel (1972) was not seen by us. On the other hand Fc-rosettes generally dominated over C3 rosettes in healthy individuals (III).

4. *Functional importance of Fc-receptors*

Fc-receptor-bearing lymphocytes, or a subpopulation of these, are thought to be effector cells in antibody-mediated lymphocyte cytotoxicity (see section VI). It has been a matter of discussion whether these cells are needed only in the induction of the reaction or if they also perform the lysis of the target cells. The finding that Fc-binding lymphocytes may be conferred with a specific cytotoxic activity following binding of antigen-antibody-complexes (Perlmann et al 1972 a, 1972 b) lends support to the notion that these cells are indeed the actual "killer cells".

It is easy to conceive functions for Fc-receptors besides being of importance for the induction of antibody-induced cytotoxicity. Such functions may include the provision of additional binding sites in the activation of lymphocytes by antigens coupled to antibodies. Fc-receptors may also function as carriers in the transport of antigen-antibody complexes by circulating lymphocytes. These aspects, which really fall outside the scope of the present review, are briefly discussed below in connection with the complement receptors (section V:C).

B. Immunoglobulin Determinants on Lymphocytes

1. *Basic data*

Antibody-secreting cells and effector cells in cell-mediated immune reactions are derived from immunocompetent progenitors, proliferating and differentiating after contact with an immunogen. Such immunocompetent cells are to be found among lymphocytes (Transplant. Rev. 1969, vol. 1). For lymphocytes to specifically recognize the antigen a receptor is needed, presumably situated at the cell surface. The only structures known to specifically recognize antigens are immunoglobulins and a fraction of lymphocytes in various species including man do indeed carry a high density of surface associated Ig. In the mouse such Ig-bearing lymphocytes have been shown to belong to the B lymphocyte population, forming precursors for antibody-secreting cells, and the capacity of the surface Ig to serve as antigen receptor has been well established (for review see Warner 1974).

Although an antigen-binding receptor is equally well needed for the initiation of cell-mediated immunity as for humoral antibody production several investigators have been unable to show Ig on the surface of T lymphocytes. These difficulties have led some investigators to deny immunoglobulins as antigen receptors on T lymphocytes or to postulate new Ig classes ("IgX") as antigen receptors on T cells (Mitchison 1969). More recently, data have accumulated that monomeric IgM is present in a low density on T lymphocytes (approximately 10^2 to 10^3 molecules per cell in the mouse) and there is now increasing evidence that antigen-binding receptors on T lymphocytes are at least partly built up by this surface Ig. The amount of Ig present on T lymphocytes is, however, too low to be shown by immunofluorescence, detection of surface Ig by this or other techniques of similar

sensitivity only revealing B lymphocytes. (For references and further discussion of antigen receptors on T lymphocytes see Marchalonis and Cone 1973, Greaves et al 1973, Warner 1974. For a different view on the nature of the T cell receptor see Simonsen 1974. For data on the possible role of β_2 -microglobulin and/or HL-A antigen structures as receptors on T lymphocytes see Transplant. Rev. 1974, vol. 21).

It is not my intention to give an extensive account of the distribution and function of Ig-bearing lymphocytes, this has been given in several reviews (Transplant. Rev. 1973, vol. 14; Warner 1972, 1974) but in the context of this thesis two important findings should be stressed:

1. Ig determinants do not constitute static structures on the cell membrane but can rather coalesce and be interiorized or shed off after cross-linking with anti-immunoglobulins or antigens (Taylor et al 1971, de Petris and Raff 1973, Ault et al 1973). This behaviour of the Ig determinants is in accord with the cell membrane being a semi-fluid compartment as proposed in the model of Singer-Nicholson (see Singer 1974). Besides being of considerable biological interest this phenomenon of "cap formation" is technically important: special precautions have to be taken (low temperature or other means for blocking cell metabolism e.g. fixation) to avoid interiorization of cell surface immunoglobulins upon treatment with anti-immunoglobulins.
2. Selective elimination of Ig-bearing cells using anti-Ig loaded columns have shown that in immunized mice the class and subclass of the surface immunoglobulins found on the B lymphocytes (antibody-forming cell precursors) are identical with those formed by the antibody-secreting cells (Wigzell et al 1972). However, in non-immunized individuals monomeric IgM is the cell surface Ig found on the majority of the B lymphocytes as studied in the mouse. Also, there is now good evidence that within a single cell clone the class of the secreted Ig may switch over from originally being IgM to later in the immune response being IgG and IgA, although there is some controversy whether this occurs in parallel or serial steps from the stem cells (see Warner 1974). The biological importance of IgD found in the membrane of a proportion of human peripheral blood lymphocytes is so far unknown (see Rowe et al 1973 a, 1973 b).

It should be emphasized that the techniques used for studying morphologically cell surface Ig on lymphocytes will only give a static picture of the occurrence of Ig-bearing cells and do not reveal the capacity of these cells to fulfill proliferation or differentiation. Also, the mere demonstration of surface associated Ig does not tell whether this immunoglobulin has the capacity to bind antigen.

2. Development of an improved procedure for detecting surface Ig on lymphocytes by the mixed antiglobulin reaction (II)

A great many investigations have now been published showing that in healthy humans a proportion of peripheral blood lymphocytes carry surface-associated Ig (Coombs et al 1969, Pernis et al 1971, Fröland and Natvig 1972 a, II, III and

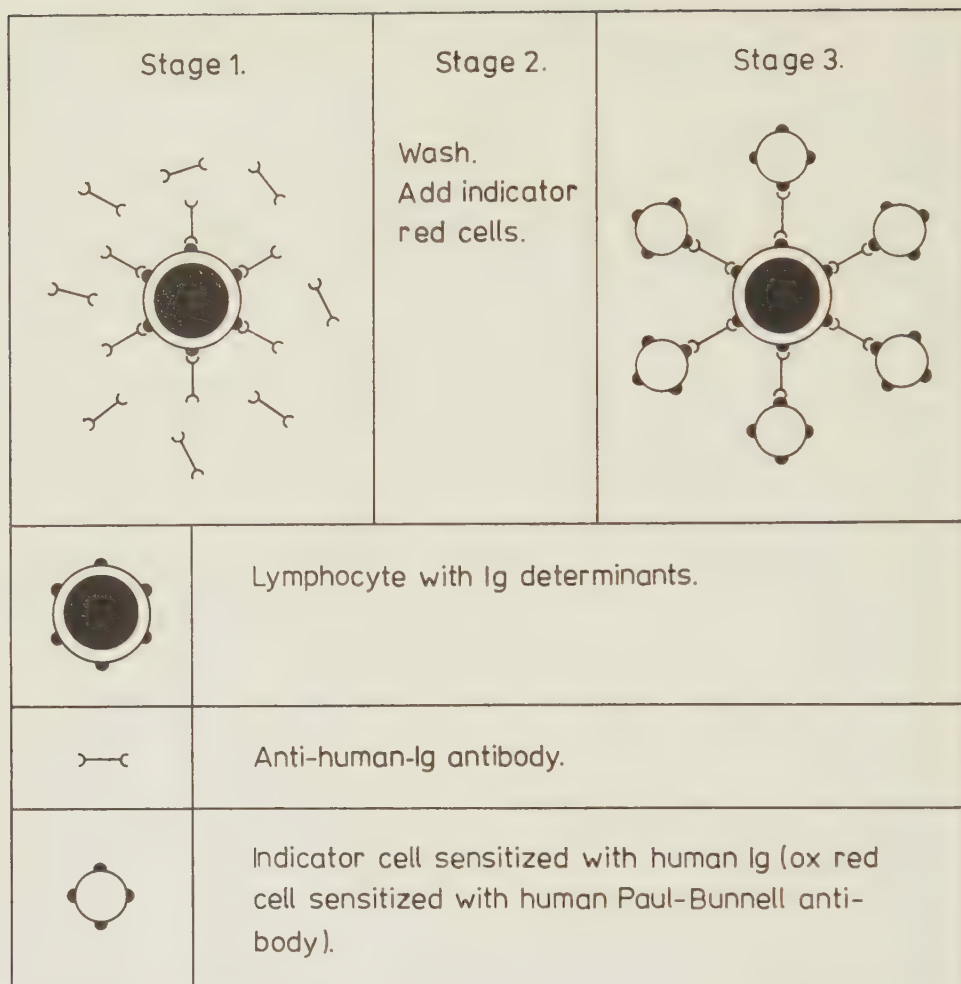


Figure 2. Detection of surface immunoglobulin on lymphocytes by the mixed antiglobulin reaction.

others). Using immunofluorescence techniques, approximately 10–30 % of peripheral lymphoid cells are stained (see also compiled data in Warner 1974).

The mixed antiglobulin reaction (MAR) (cf. Figure 2 and Coombs and Franks 1969 p. 201 ff.) was originally introduced by Coombs for the detection of antibodies in human sera against human platelets and leucocytes (Coombs et al 1956, Chalmers et al 1959). Later, this technique was applied for the detection of surface Ig determinants on lymphoid cells in man (Coombs et al 1969) and other species (Coombs et al 1970 a, 1970 b, Aitken et al 1972).

In these investigations it soon became evident that freshly prepared live lymphocytes only weakly displayed surface Ig determinants as shown by the MAR. Such Ig determinants could be shown, however, after having stored the lymphocytes over liquid nitrogen (Coombs et al 1970 b). Even after this treatment the percentage reactive cells was rather low (2–8 % of peripheral blood lymphocytes in man,

Coombs et al 1969) compared to results obtained with immunofluorescence techniques. Higher values were seen by Litwin et al (1973) using a double layer mixed antiglobulin method.

It was thought that the poor reactivity of freshly prepared cells might be due to a coat of a membrane-associated substance. The finding (II) that simply storing the freshly prepared lymphocytes in saline at $+4^{\circ}\text{C}$ increases the percentage reactive cells in the MAR could be explained by the elution of such a coat. This procedure, however, was not suited for routine use because of difficulties in standardization. Instead, a new procedure for pretreatment of lymphocytes for the detection of surface Ig was described (II). This method includes fixation of the lymphocytes in a buffered formaldehyde solution, as described originally by Karnovsky (1965) for electron microscopy, and leaves a clean unclumped suspension of lymphocytes free of contaminating red cells.

This simple method offers several advantages over the earlier technique using low temperature (-180°C) storage. First, and most important, lymphocytes fixed in formaldehyde were more reactive in the MAR, i.e. more and stronger rosettes were formed, and the results with lymphocytes so treated were well on a level with those obtained by other investigators using immunofluorescence techniques. Second, and of considerable practical importance, the fixed lymphocytes could be stored for several days without change in their reactivity in the MAR (II, Table 1). The procedure of formaldehyde fixation has now been found to be generally applicable to the lymphocytes from several different species (Escajadillo et al 1974) with results superior to those obtained with low-temperature stored cells. Further experience (I, III) has shown that following treatment with formaldehyde the lymphocytes can no longer form Fc- or C3-rosettes, nor bind sheep RBC. This ensures that irrelevant rosettes are not formed in the mixed antiglobulin reaction.

Besides the treatment of the lymphocytes the handling of the sensitized indicator red cell system was critical for the outcome of the MAR (II). Thus, when using non-formalinized frozen lymphocytes the cell concentration of the indicator red cell suspension must not go below a certain limit (in the present system 0.8 %) to identify an optimal number of Ig-bearing lymphocytes in chronic lymphatic leukaemia (CLL). In contrast, non-formalinized lymphocytes from healthy donors reacted efficiently with considerably more dilute red cell suspensions as did formaldehyde-treated lymphocytes from normal donors as well as CLL patients. The reason for this phenomenon is unclear but might be related to differences in the charges of the cell surfaces of lymphocytes treated in different ways and possibly also to the low density of Ig determinants reported on leukaemic lymphocytes (Aisenberg and Bloch 1972).

3. Frequency and Ig class distribution of immunoglobulin determinants on human lymphocytes from healthy donors and patients with chronic lymphatic leukaemia (II, III)

In paper II mainly technical aspects of the MAR are dealt with. However, when comparing lymphocytes from healthy donors and patients with chronic lymphatic

leukaemia (CLL) a consistent finding, irrespective of the pretreatment of the lymphocytes, was an increased percentage of reactive lymphocytes in the CLL group. In the standard procedure using low temperature (-180°C) stored lymphocytes from 14 healthy donors and 17 CLL patients, a mean of 12 % and 52 % Ig-bearing cells, respectively, were shown. This should be contrasted with the mean of 34 % and 86 %, respectively found in paper III when formaldehyde-treated lymphocytes from 20 normal donors and 11 CLL patients were investigated.

The indicator system used in these investigations (II and III) was designed to detect Fab determinants as well as μ -chain determinants on the lymphocytes, based on earlier experiences of the MAR on human lymphocytes (Coombs et al 1969). The percentages of reactive lymphocytes in normal donors as detected in the "formaldehyde version" of the MAR were now well on a level with those obtained by others using immunofluorescence (cf. preceding section).

Animal experiments have provided confidence that lymphocytes bearing a high density of surface Ig are B lymphocytes (antibody-secreting cell precursors). The evidence that this applies also to Ig-bearing lymphocytes in man is more indirect, but is consistent with findings in immune deficiency diseases and with the distribution of Ig-bearing lymphocytes in various lymphoid organs (see Transplant. Rev. 1973, vol. 16). In this context it is of interest to compare the results obtained in the MAR with those of other lymphocyte markers studied in parallel (III, Tables II and V). Sheep RBC-rosettes, which are thought to mark T lymphocytes (see section V:D), varied inversely with MAR-rosettes, the sum of these two tests never appreciably exceeding 100 %. This was so also in CLL patients with a vast dominance of Ig-bearing cells. These findings were thus consistent with the notion that surface Ig and affinity for sheep erythrocytes are markers for different lymphocyte subpopulations in man. Among the "B cell reactions" most individuals (normal donors as well as CLL patients) had more lymphocytes reacting in the MAR than in the Fc- or C3-rosetting tests. However, in 4 normal donors there were more Fc-rosettes than Ig-bearing lymphocytes. These results indicate that surface Ig occurs (or at least may occur) independently of the other markers studied. Unfortunately, the MAR technique did not allow studies of mixed rosettes as performed in the sheep RBC/ Fc- and sheep RBC/ C3-rosetting systems to evaluate whether several markers co-occurred on single cells.

As discussed in paper II p. 330 and paper III p. 534 our finding of a high proportion of Ig-bearing lymphocytes in CLL is in accordance with independent observations by others (see also Grey et al 1971, Transplant. Rev. 1973, vol. 16, Ternynck et al 1974, Mellstedt and Pettersson 1974). It is tempting to presume that these Ig-bearing lymphocytes represent clones of tumour cells in analogy to clonal plasma Ig in multiple myeloma or Waldenström's macroglobulinaemia. Evidence in support of this presumption has emerged, showing that in a majority of CLL patients the lymphocyte surface Ig is restricted to one particular type of heavy and light chains (Grey et al 1971, Preud'homme and Seligmann 1972 b). As observed in normal lymphocytes (see Warner 1974) μ -chain specificities are more often represented on the cell surface of CLL lymphocytes than are γ or α specificities, and likewise kappa chains predominate over lambda chains (Grey et al 1971,

Preud'homme and Seligmann 1972 b, Fröland and Natvig 1972 b, II, Jondal et al 1973, Ternynck et al 1974). A recent report (Kubo et al 1974) indicates that IgD may be the major Ig class present on CLL lymphocytes and that a considerable proportion of CLL cells may bear IgD and IgM on their surface. These studies thus clearly show that the immunoglobulin present on the lymphocyte surface consists of both heavy and light chains. An interesting observation (II) was the finding that comparing the lymphocytes of 2 CLL patients one exhibited mainly Fab determinants, the other mainly μ Fc determinants indicating that the exposure of the Ig molecules on the cell surface may be different in different lymphocyte populations. Very recently Merler et al (1974) have provided evidence that the immunofluorescent staining of human B lymphocytes with certain antisera to IgM immunoglobulin might not be due to the presence of IgM on these lymphocytes. Rather, it could be caused by interaction of these antisera with carbohydrate present on the surface of the lymphocytes and cross-reacting with the carbohydrate of IgM immunoglobulin. Further studies are needed to evaluate this observation which might possibly be one reason why different authors have found such wide variations in the proportion of μ -chain positive cells among human Ig-bearing lymphocytes (see Warner 1974, p. 112).

A critical point is whether the Ig shown on the cell surface constitutes an integral part of the lymphocyte cell membrane or is bound to the cell from without. In support of the former concept Preud'homme and Seligmann (1972 b) were able to show *de novo* synthesis of surface-associated Ig in trypsinized CLL lymphocytes. In the present investigation (II) a different approach was chosen to see whether the lymphocytes were coated with antibodies from without: CLL lymphocytes were treated with fresh human serum and then tested for opsonic adherence to macrophages *in vitro*. No such adherence occurred, suggesting that the CLL lymphocytes as taken from the patients were not coated with opsonizing or complement fixing auto-antibodies. In contrast, CLL lymphocytes treated with human anti-HL-A antiserum and human complement formed opsonic rosettes around the macrophages. It should be mentioned that the innate Ig of the lymphocyte membrane may have anti-Ig activity and bind additional Ig molecules to the cell surface (Preud'homme and Seligmann 1972 a). Further possibilities of Ig binding occur via the Fc-receptors present on a majority of CLL lymphocytes or via direct binding of auto-antibodies. Evidence against the binding of such external Ig to the lymphocytes was the finding that γ Fc determinants did not occur in any of 10 tested CLL patients (II).

C. Receptors for Complement

Antigen-antibody complexes which have reacted with complement factors including C3 are known to adhere to primate red cells and to certain non-primate platelets, so called "immune adherence" (R. A. Nelson 1953. For review see D. S. Nelson 1963, Coombs and Franks 1969, p. 216). This reaction has been used as an *in vitro* test for antigen-antibody-complement complexes (cf. Coombs and Franks 1969, p.

217, Nishioka 1971) but the possible *in vivo* importance of the phenomenon is largely unknown.

Receptors for antigen-antibody-complement complexes were shown on phagocytic cells such as monocytes and granulocytes in a number of different species including man (Huber et al 1968, Lay and Nussenzweig 1968, Henson 1969, Henson 1972). It is considered that the binding via such complement receptors of complement-carrying particles (e.g. bacteria, antigen-antibody complexes) constitutes an important phagocytosis-promoting mechanism, the bound complement acting as an opsonin (Lay and Nussenzweig 1968).

The presence of receptors for complement on the surface of certain lymphocytes was first shown by Uhr (1965) and Uhr and Phillips (1966) in the guinea-pig. Further studies, i.a. by Nussenzweig and his group (reviewed in Nussenzweig and Pincus 1972, Nussenzweig 1974) revealed that a subpopulation of peripheral blood lymphocytes of mouse and man (Bianco et al 1970, Ross et al 1973 a, b) also have receptors for complement, probably reacting with bound C3.

1. *Complement receptors on human lymphocytes (III)*

The present investigation (III) was undertaken to study the frequency of complement-receptor lymphocytes (CRL) in the peripheral blood of healthy humans and patients with chronic lymphatic leukaemia (CLL) and to see how the complement receptors were distributed in relation to other lymphocyte surface markers. CRL in the peripheral blood of 20 normal individuals and 11 patients with chronic lymphatic leukaemia were demonstrated with a rosetting technique using as indicator cells ox red cells sensitized with rabbit IgM-antibody and treated with human complement (zymosan-treated whole human serum). This reagent contains C1-3 but lacks C5-9 (see Pepys and Butterworth 1974).

In *healthy humans* 8-31 % (mean 14.7 %, SE 1.2) of peripheral lymphocytes carried the complement receptor (III). Among the "B cell reactions" (Fc rosettes, complement rosettes, mixed antiglobulin reaction) the percentage of CRL was generally lower than that of the other two reactions. Thus, in 10 of the donors the percentage of Fc rosettes was twice or more of the complement rosettes and in 15 donors there were approximately two to five times as many Ig-bearing lymphocytes as CRL. In healthy humans several investigators (Michlmayr and Huber 1970, Bianco et al 1970, Pincus et al 1972, Ross et al 1973 b) have found CRL to constitute a minority (20 % or less) of peripheral blood lymphocytes. Slightly higher values (25-33 %) were seen by Jondal et al (1972) who might, however, have included some monocytes in their lymphocyte suspensions.

Evidence was presented (III) that complement-receptor lymphocytes generally do not bind sheep RBC (as discussed in section V:D there is considerable evidence that the sheep RBC receptor is a marker for human T cells). This was done in a rosetting test using a mixture of fluoresceinated sheep RBC and non-fluoresceinated, complement-carrying ox red cells as the indicator cells. Four normal donors were tested and in all at most 3 % of all rosettes formed were mixed, indicating that, with few exceptions, separate lymphocyte populations carry the receptors for sheep

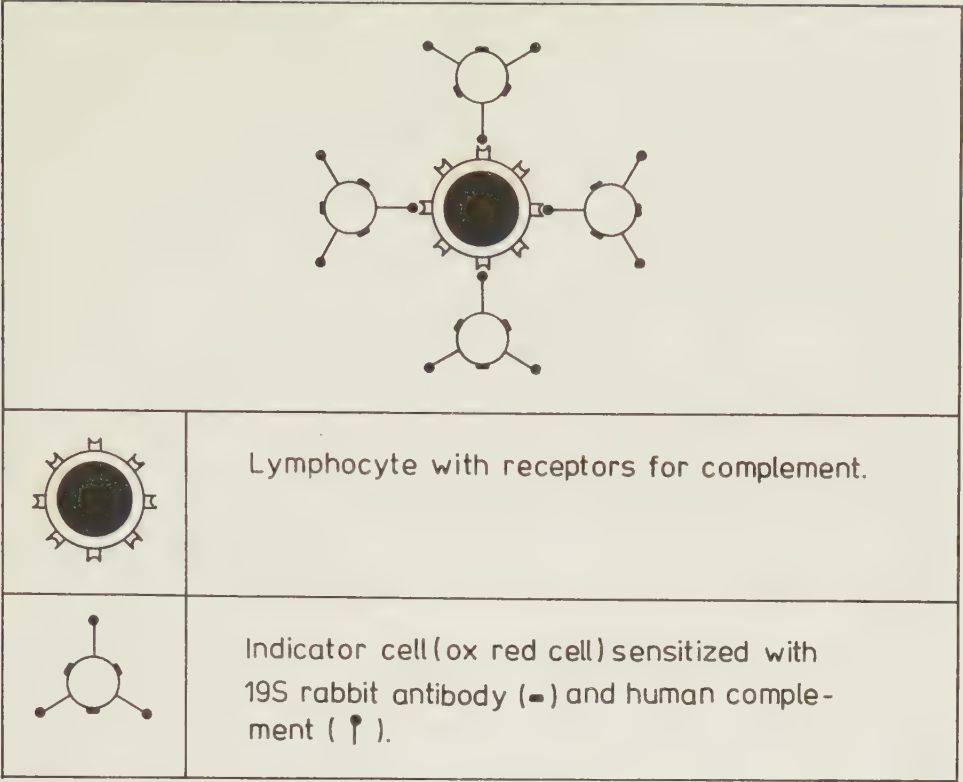


Figure 3. Detection of complement receptors on lymphocytes.

RBC and complement. These results are in accordance with those of Jondal et al (1972) who also used a mixed rosetting technique to show that CRL and sheep RBC-binding lymphocytes in man are not identical. The possible significance of the finding of certain mixed rosettes in the sheep RBC-C3-rosetting test is commented upon below (section V:C:3).

In *chronic lymphatic leukaemia* 21–97 % (mean 56.4 %, SE 7.7) of peripheral lymphocytes bound complement-bearing indicator cells (III) and thus the majority of the patients had a considerable increase in the relative number of CRL. Still, the number of CRL was consistently lower or, in two patients, approximately the same as the Ig-bearing lymphocytes. In two patients the CRL comprised only one-fourth of the Ig-bearing cells. These proportions between CRL and Ig-bearing lymphocytes are similar to the situation in the normals, although the absolute figures are on a higher level in the CLL group. As opposed to the finding in the normals there was no consistent relation between Fc-binding lymphocytes and CRL in chronic lymphatic leukaemia, some patients being high in the complement rosettes and low in the Fc-rosettes and vice versa. The low frequencies (mean 11.8 %) of sheep RBC rosettes in the CLL group (III) are compatible with the observation in the normals that the receptors for complement and for sheep RBC are expressed on separate lymphocyte populations.

Differences in the technology used, together with the use of complement from different species, make it hard to evaluate the low frequencies of CRL in CLL patients seen by Michlmayr and Huber (1970), Nishioka (1971) and Jondal et al (1973) in comparison with the increased values seen by us (III) and by others (Pincus et al 1972, Shevac et al 1972, Ross et al 1973 b). It should be emphasized that even in CLL materials with an overall increase in the percentage of CRL there have been a few patients with normal or even subnormal levels of CRL. So far, the differing levels of CRL in various CLL patients have not been shown to correlate with the course of the disease.

Pincus et al (1972) as well as Ross et al (1973 b) noted that CLL lymphocytes, in contrast to normal lymphocytes, reacted better with mouse complement than with human complement. They supposed that this was due to the occurrence of two receptors with different species-specificity on normal and CLL lymphocytes. Recently, lymphocyte receptors with specificities for various fragments of the C3 molecule have been described (Ross et al 1973 a, Eden et al 1973 b) emphasizing the technique for complement-loading on the indicator cells, rather than the species origin of the complement used. These entities are discussed in more detail in the following section.

2. Specificity of complement receptors

The demonstration of receptors for complement on phagocytes or lymphocytes has generally been performed by rosette-formation procedures using red cells bearing 19S antibodies and complement as the indicator cells. Complement factor C3 is necessary for the binding (Lay and Nussenzweig 1968, Bianco et al 1970) but some peculiar differences between various experimental models have been seen.

Thus, divalent cations (preferentially Mg^{2+} ions) are necessary for complement dependent rosette-formation with phagocytic cells in the mouse (Lay and Nussenzweig 1968) and in the rabbit (Henson 1969) using homologous complement. On the other hand lymphocytes can form complement rosettes in the absence of divalent cations (Michlmayr and Huber 1970, Nussenzweig et al 1971). Likewise most investigators find complement binding to human monocytes and granulocytes in the absence of Mg^{2+} ions (Henson 1969, Nussenzweig et al 1971) but under certain experimental conditions Ross et al (1973 a) could distinguish between the complement receptor on human monocytes and lymphocytes by the blocking with EDTA of the former. Similar findings were earlier reported by Michlmayr and Huber (1970). Henson (1969) and others also observed that granulocytes of certain species, including man, bound homologous as well as certain heterologous complements. In contrast, the granulocytes of rabbit and guinea-pig reacted only weakly with complement from certain other species, homologous complement always being effective.

These rather incoherent results have been reviewed here because they indicate that the complement binding receptors on various phagocytic cells and on lymphocytes, are not all identical and that this is not due solely to differences in the species-specificity of these receptors. For human CLL lymphocytes mouse comple-

ment has been considered to detect many more CRL than does human complement and this has been considered to be due to differences in the species-specificity of the receptors on normal and leukaemic lymphocytes (Ross et al 1973 b). These observations can now partly be explained on a molecular basis since it has been shown that normal human lymphocytes have receptors for (at least) two different determinants of the C3 molecule. First, one receptor binding fixed C3 (=C3b) and resembling the primate erythrocyte immune adherence receptor and second, one receptor binding C3b which has been altered by the action of the C3b-inactivator (Ross et al 1973 a, Eden et al 1973 b). In contrast, CLL lymphocytes generally lack the former of these receptors (Ross et al 1973 a). As prepared in the present investigation (III) the complement bearing ox red cells will detect the receptor for C3b-inactivator-treated fixed C3b (see Pepys and Butterworth 1974). Differences in the degree of fragmentation of the C3 molecule after binding to the indicator red cell membrane, rather than differences in species-specificity, might thus explain the differing results obtained in CLL patients using human complement (as purified components) and mouse complement (as whole serum), (see also Dierich et al 1974). Accordingly, Jondal et al (1973) using whole serum as complement source found no difference between human and mouse complement for the detection of CRL in CLL patients.

Theophilopoulos et al (1974 a) have shown that human lymphoblastoid cell lines (*e.g.* Raji cells) bind soluble C3, in addition to C3b, probably by the same receptor site, or by two closely associated sites on the cell membrane. In contrast, human peripheral blood lymphocytes only bound C3b. In a later investigation (Theophilopoulos et al 1974 b) evidence was presented that in human lymphoblastoid cell lines surface Ig, receptors for Fc of IgG, receptors for C3-C3b and receptors for C3d (*i.e.* C3b-inactivator - treated C3b) constitute separate sites on the cell membrane. However, the binding of antigen-antibody-complement complexes to the cell surface occurred via the complement receptors without involving Fc-receptors. In contrast, non-complement-bearing immune complexes bound to cell surface Fc-receptors. Whether this finding applies to human peripheral lymphocytes as well is not known but in the rosetting experiments experience shows that C3 - rosettes are much tougher than Fc-rosettes, indicating a high affinity between the complement receptor and the corresponding ligand.

The complement receptor present on human granulocytes seems to have the same specificity as the immune-adherence receptor on human red cells, binding fixed, unaltered (but not altered) C3b (Ross et al 1973 a, Eden et al 1973 b). Besides the classical immune-adherence receptor binding C3b, Cooper (1969) has demonstrated immune-adherence to human 0 erythrocytes mediated by complement factor C4. Ross et al (1973 a) mention that receptors for C4 occur on granulocytes. The question naturally arises whether receptors for complement factors other than C3, as for example C4, may occur on lymphocytes as well.¹

¹ Since this was written, V. A. Bokisch and A. T. Sobel (J. exp. Med. 140, 1336-1347, 1974) have described a receptor for C4 on human peripheral non-T lymphocytes and cultured lymphoblastoid cells.

3. Distribution of complement receptors in relation to other lymphocyte surface markers

Whereas complement receptors were shown on nearly all monocytes and neutrophils of an individual, only a minor subpopulation of peripheral blood lymphocytes carried complement receptors (Henson 1969, Lay and Nussenzweig 1968, Huber et al 1968). Nussenzweig and collaborators (see Nussenzweig and Pincus 1972) presented evidence that in the mouse CRL are identical with most or all Ig-bearing or B lymphocytes. Our own results (III) as well as those of others (Jondal et al 1972, Yata et al 1973 b) indicate that in man the C3-receptor does not occur, more than exceptionally, on sheep RBC-binding lymphocytes and therefore is probably a marker of non-T lymphocytes. The finding (III) of up to 3 % mixed sheep RBC-C3-rosettes among rosetting normal lymphocytes could, however, be taken as evidence that complement receptors may be carried by certain T lymphocytes as well. In fact, a case of CLL has recently been described in which the lymphocytes were said to simultaneously carry receptors for sheep RBC (T cell marker) and for complement (Shevac et al 1973). As a broad generalization, however, it could be said that in man CRL are non-T cells.

Our data (III) as well as those of others (Schiff et al 1974, Perlmann et al 1974) show that CRL are not identical with Ig-bearing lymphocytes in humans, whereas Nussenzweig and Pincus (1972) found very close association between these markers in the mouse. Some results, however, point to a not absolute homology between Ig-bearing lymphocytes and CRL even in the mouse (Nussenzweig et al 1971). In man a significant proportion of Ig-positive lymphocytes does not bear detectable C3 receptors (Ross et al 1973 b, III) and conversely some CRL lack surface Ig (Ross et al 1973 b). Jondal et al (1972), however, found close homology between CRL and surface Ig in healthy humans. Experiments by Bianco and Nussenzweig (1971) in the mouse and by Abrahamsohn et al (1974) in man, indicated that surface Ig and receptors for complement occur as separate entities on a single lymphocyte.

In the mouse Eden et al (1973 a) have found a majority of CRL to carry the Fc-receptor as well. Our data (III) collected in the normals do not allow conclusions whether C3 and Fc-receptors occur on the same or different cell populations, but in the CLL group these receptors were expressed independently of each other. It should be emphasized, as has been done earlier in this text, that the various receptors on CLL lymphocytes are not necessarily identical with those on normal lymphocytes. Consequently, conclusions concerning the distribution of various markers in relation to each other, as observed in CLL lymphocytes, may not be valid for normal lymphocytes.

4. Possible biological role of complement receptors on lymphocytes

The concept that complement receptors on phagocytic cells have a central role in opsonization procedures has been dealt with above and seems to be well established. As to the possible biological role of the complement receptor on lymphocytes, Dukor

and Hartman (1973) have put forward the hypothesis that this receptor receives a "second signal" when B lymphocytes are activated by thymus dependent or thymus independent antigens, pointing also to the fact that many thymus independent antigens can serve as B cell mitogens and have the capacity to activate C3 via the alternate pathway. Evidence against a general role for the C3 receptor in B cell activation by thymus-independent antigens or B cell mitogens has been presented by Janossy et al (1973) and it should be stressed that other theories of B cell activation, for example the "one non-specific signal"-hypothesis of Coutinho and Möller (see Coutinho and Möller in press), give no major role for the C3 receptor in the activation process.

Another possibility would be that C3 receptors could serve as mediators for the induction of lymphocyte cytotoxicity for target cells bearing antigen-antibody-complement complexes. Studies by Perlmann et al (1973 a, b) show, however, that although an efficient contact is established between lymphocytes and C3-bearing target cells, no cytolytic reaction is initiated. This is in contrast to the efficient induction of lymphocyte cytotoxicity by IgG antibodies on target cells (cf. section VI of this thesis).

Although the biological function of complement receptors on lymphocytes has not been unequivocally established it seems probable that they might be of importance in some stage of B cell activation, possibly by clustering more efficiently certain B lymphocytes to antigen-antibody-complement aggregates. Nussenzweig and collaborators (Nussenzweig et al 1971, Nussenzweig and Pincus 1972) have emphasized the role of CRL in the concentration of antigen to follicular areas in lymphoid organs. The objection to a general role for the complement receptor in immune response, that only a fraction of B cells show this receptor, seems less valid. It might well be that the receptor is expressed only during certain stages of B lymphocyte development (see Möller 1974). For further discussion of the possible functions of the complement- and Fc-receptors on lymphocytes see Nussenzweig (1974).

D. Receptors for Sheep Erythrocytes

1. Basic data on determinants characterizing thymus-dependent lymphocytes

Processing of lymphoid cell precursors within the thymus (as studied in certain strains of inbred mice) is accompanied by changes of the cell membrane including the appearance of new surface antigens, the theta and thymus-leukaemia (TL) antigens (Boyse et al 1966). During maturation of the thymic lymphocytes the density of these antigens is reduced, with a concomitant rise in the density of H-2 transplantation antigens. The TL antigen is ultimately lost, probably before the cells are exported from the thymus to the peripheral lymphoid organs. The theta antigen is retained on the exported cells and is considered to characterize peripheral lymphocytes derived from the thymus, i.e. T lymphocytes (for review see Raff 1971).

Until recently no markers for T lymphocytes were known in man. In 1970 several workers described that a considerable fraction of human peripheral blood lymphocytes would bind unprepared sheep erythrocytes (sheep RBC) (Coombs et al 1970 c,

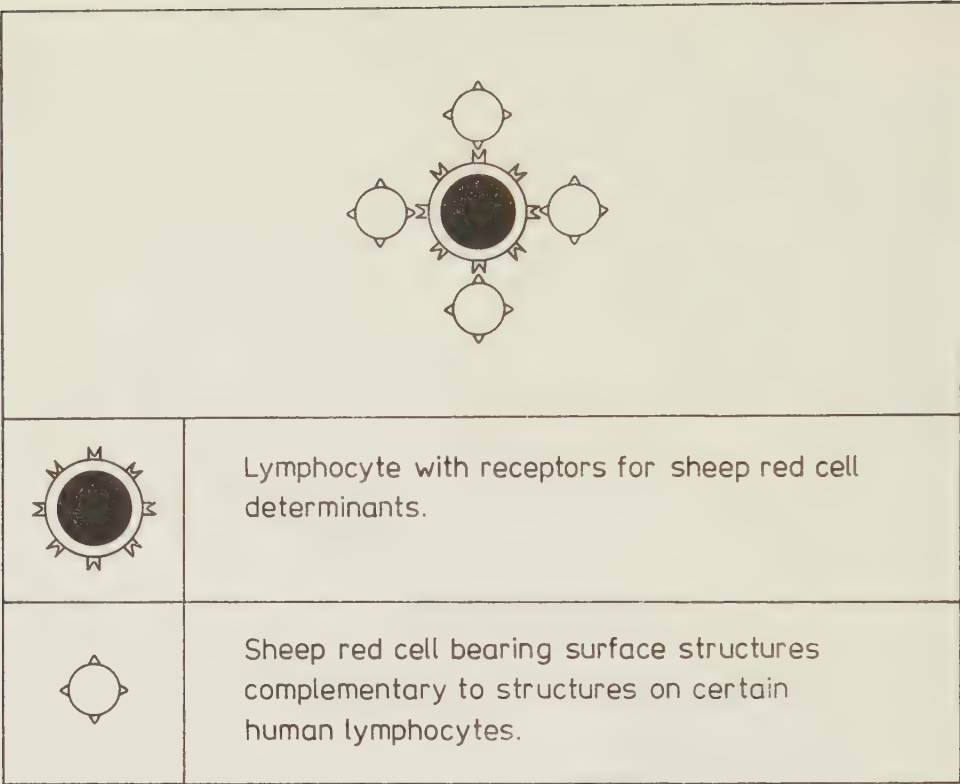


Figure 4. Detection of receptors for sheep erythrocytes on human lymphocytes.

Brain et al 1970, Lay et al 1971) and evidence was presented that this binding did not involve Ig on the reacting cells. Later evidence has accumulated that the binding of sheep RBC might be a marker for human T lymphocytes. The evidence for this can be summarized as follows:

1. A high proportion (up to 100 %) of lymphoid cells prepared from the human thymus bind sheep RBC (Lay et al 1971, Wybran et al 1972, Jondal et al 1972).
2. The tissue localization of sheep RBC-binding lymphocytes in lymph nodes, spleen and thymus coincides with the earlier concept of T cell distribution (Silveira et al 1972).
3. Most evidence, collected along several lines, has indicated that human Ig-bearing lymphocytes do not bind sheep RBC (Coombs et al 1970 c, Brain and Gordon 1971, Papamichail et al 1972, Jondal et al 1972, Fröland 1972) although a single exceptional report of inhibition of sheep RBC rosettes by anti-Ig sera of various specificities has been given (Wybran et al 1972).

More recently additional markers for human T lymphocytes have been described. Thus several groups have now produced antisera against human T cell antigens (Aiuti and Wigzell 1973, Williams et al 1973, Wortis et al 1973, Yata et al 1973 a, b). Hammarström et al (1973) have shown selective binding of *Helix pomatia* haemagglutinin to a population of (neuraminidase-treated) human lymphocytes,

probably T cells. So far these new markers for human T cells have found more restricted applications compared to the sheep RBC rosetting reaction. Antisera to human T cell antigens do not always indicate the same lymphocyte populations as does the sheep RBC rosetting reaction (see Yata et al 1973 a), and some doubt may be raised as to the validity of the sheep RBC receptor as a strict marker of human T lymphocytes (see further discussion in sections V:D:3 and V:E of this thesis).

2. Nature of the binding between human lymphocytes and sheep erythrocytes

Already the original description of the sheep RBC rosetting phenomenon indicated that this reaction is not mediated by surface-associated immunoglobulins on the lymphocytes (Coombs et al 1970 c). Also, it is hard to see how in non-immunized humans 50 % or more of peripheral blood lymphocytes can bear Ig with specificity for sheep RBC. Rather there seems to be a structural complementarity between certain human lymphocytes and sheep RBC. The operative structures on the lymphocytes are not well characterized but are probably of protein nature, being destroyed by proteolytic enzymes, e.g. trypsin (Jondal et al 1972, Fröland 1972). The effect of trypsin treatment of the sheep RBC has been more variable (Jondal et al 1972, Bianco et al 1973). In contrast, the reaction is facilitated after treatment of sheep RBC with neuraminidase (Bianco et al 1973, Hallberg, unpublished observations), probably due to removal of negatively charged groups on the cell surface. We have found that the reactivity of the sheep RBC also increases with ageing for several weeks of the stored sheep blood (Hallberg, unpublished observations), possibly due to a mechanism similar to that of neuraminidase. In the present investigation sheep ACD blood stored for at most one week was used without treatment with neuraminidase.

Earlier experiments with the sheep RBC rosetting reaction have established 2 technically important points which were confirmed in the present investigation (III):

First, only *live* lymphocytes form sheep RBC rosettes (Coombs et al 1970 c). In the present investigation the viability of the cells was checked with toluidine blue dye, only live cells being counted in the mounted cell suspensions.

Second, the sheep RBC rosettes are very fragile and the rosette formation has a pronounced temperature dependence. We have been using the two-stage procedure of Lay et al (1971) for preparation of rosettes, keeping the cells at +4°C after centrifugation. In accordance with earlier observations (Lay et al 1971) it was seen that increasing the temperature after the rosettes had formed led to dissociation of lymphocytes and sheep RBC. Further technical aspects on the sheep RBC rosetting reaction may be found in Bach (1973) and in a WHO report (1974).

3. Sheep RBC – rosettes formed with the lymphocytes from healthy donors and patients with chronic lymphatic leukaemia (I, III)

The sheep RBC-rosetting reaction was used as a possible marker of human T cells in an investigation of the lymphocytes from 20 healthy donors and 11 CLL patients

(III). In addition to this straight-forward rosetting reaction experiments were undertaken in a limited number of healthy donors to study whether sheep RBC would bind to lymphocytes bearing receptors for Fc of IgG or for complement (I, III). In this type of experiments the lymphocytes were centrifuged with a mixture of indicator cells, of which the sheep RBC had been stained with fluorescein, and the rosettes scored as unmixed or mixed by counting in both phase contrast and UV light.

In *healthy donors* 44–72 % (mean 55.7 %, SE 2.2) of live lymphocytes reacted in the sheep RBC rosetting reaction (III). Similar values have been found by Jondal et al (1972) and Stjernsward et al (1972) whereas others (Fröland et al 1972, Fröland 1972, Wybran et al 1972, 1973 b) have found mean values below 30 % reactive lymphocytes. Differences in the techniques used probably account for the major part of these discrepancies (cf. Bach 1973, WHO report 1974). The observed percentages of sheep RBC rosettes in the individual donors (III) was compatible with the assumption that this reaction marks a cell population distinct from those characterized by the "B cell reactions" (Fc rosettes, C3 rosettes, mixed antiglobulin reaction). Thus, the sum of sheep RBC rosettes and any of these 3 reactions did not appreciably exceed 100 % in any of the donors. Direct evidence that there is no major "overlap" between sheep RBC-binding lymphocytes and lymphocytes binding complement was found in the mixed rosetting experiments described above, in which at most 3 % mixed rosettes were found in 4 healthy donors (III). Similar experiments using mixtures of sheep RBC and IgG-coated ox RBC gave more variable results. Thus in one investigation (III) at most 4 % mixed rosettes were found in 4 healthy donors, whereas in a separate test (I) 2 healthy donors had 8 and 21 % mixed rosettes respectively. Although these results indicate that lymphocytes bearing receptors for complement or for Fc of IgG generally do not have receptors for sheep RBC as well, the finding of certain lymphocytes bearing both B and T cell markers is of considerable interest. Dickler et al (1974) have studied receptors for aggregated IgG and surface Ig determinants on lymphocytes binding sheep RBC. They found that a mean of 3.6 % of peripheral lymphocytes in normal donors bore both T and B cell markers, a value very similar to that reported in paper III. Bentwich et al (1973) have shown that human lymphocytes treated with neuraminidase form more and stronger rosettes with sheep RBC than do untreated lymphocytes. In addition some of these lymphocytes displayed Fc-receptors as well as receptors for sheep RBC. In the mouse, Yoshida and Andersson (1972) have shown Fc-receptors on activated mouse T lymphocytes, whereas Smith and Haegert (1974) found at most 5 % mixed sheep RBC-Fc or sheep RBC-C3 rosettes on PHA-transformed human lymphocytes.

Thus, although a great body of data indicates that the sheep RBC rosetting test in many situations may be used as an indicator of human T lymphocytes, it is equally clear that sheep RBC-binding lymphocytes do not all belong to a single population. Results in the sheep RBC rosetting tests are much dependent upon the particular technique used and with certain modification it seems as if some Fc-binding lymphocytes are included as well. Part of these difficulties may be due to differences in the surface charge of various lymphocyte populations (cf. Mehrishi

and Zeiller 1974). In my opinion it is premature to equalize sheep RBC-rosetting human lymphocytes with T lymphocytes as defined from mouse experiments (see also section V:E of this thesis).

All *CLL patients* had subnormal levels (mean 11.8 %, SE 3.4) of sheep RBC-binding lymphocytes (III). As mentioned above these patients had increased proportions of lymphocytes bearing "B cell markers" and this was thus reflected by a simultaneous decrease in sheep RBC-binding lymphocytes. As seen in the healthy donors the sum of sheep RBC rosettes and any of the 3 "B cell reactions" did not appreciably exceed 100 %. Again, this is compatible with the notion that the sheep RBC reaction marks a separate lymphocyte population and that the leukaemic lymphocytes are non-T cells. Investigations by other groups (Papamichail et al 1972, Wybran et al 1973 a, Ross et al 1973 b) have also shown that the sheep RBC-binding lymphocytes are generally few or absent in CLL. Exceptional cases of CLL with very high levels of sheep RBC-binding lymphocytes have been described (Seligmann et al 1973, Dickler et al 1973, Brown et al 1974) and are thought to be examples of T cell malignancies. That the low frequencies of sheep RBC-binding lymphocytes seen in most cases of CLL should be due to a loss of the receptor seems unlikely particularly on the basis of the data in (III) showing that 3 different B cell markers are all more or less increased in these patients.

E. Inter-Relations of Lymphocyte Surface Markers. "Unaccounted" Cells

From the discussion in the previous sections (V:A-D) it can be concluded that in *healthy humans* the 4 lymphocyte markers studied in the present investigation may be roughly divided into two groups:

1. *Receptors for sheep RBC*, thought to occur on human T lymphocytes.
2. *Surface Ig, receptors for Fc of IgG, receptors for complement*, thought to occur on non-T lymphocytes.

Among the non-T cell markers, studied in normal donors (III), surface Ig generally accounted for more lymphocytes (mean 34 %) than did either receptors for Fc (mean 26 %) or receptors for complement (mean 15 %). A mean of 10 % of lymphocytes in healthy donors could not be accounted for as Ig-bearing or sheep RBC-binding lymphocytes (III, Table II). In isolated cases up to 27 per cent of the lymphocytes were "unaccounted" (III, Table II). Similar observations have been made by Mellstedt and Pettersson (1974) in 7 normal donors. At least part of these unaccounted cells may refer to a population of lymphocytes bearing receptors for Fc and C3, but lacking surface Ig and receptors for sheep RBC, and thought to function as effector cells in antibody-induced lymphocyte cytotoxicity (see below section VI:C:1).

The uneven distribution of the 3 markers thought to characterize non-T lymphocytes might be due to a series of functionally different lymphocyte populations within the non-T cell population. However, it could be that Ig-bearing lymphocytes express Fc- and/or C3-receptors only during part of their life span (cf. Möller 1974)

and that the observed discrepancies thus reflect non-T lymphocytes of various degrees of maturation. Of course, these two alternatives are not mutually exclusive.

Again, it was observed that receptors for Fc of IgG (and possibly also receptors for complement) thought to characterize non-T lymphocytes, might occur on a minority of sheep RBC-binding lymphocytes (I, III). Whether these lymphocytes should be characterized as T lymphocytes bearing "B cell markers" or if they belong to a non-T cell population is not clear at the moment. Evidence has emerged that the sheep RBC-receptor does not always coincide with other (antigenic) markers of human T lymphocytes (Yata et al 1973 a). It may be wise not to consider the sheep RBC receptor as a strict marker of human T lymphocytes, although, generally, it may be used as an indicator of this lymphocyte population.

It is clear that, especially in persons with high numbers of so-called "unaccounted" cells, it would be of interest to analyze in detail the occurrence of cell surface markers on individual cells. Unfortunately, the mixed antiglobulin reaction did not allow us to study the distribution of surface Ig in relation to other markers.

In *patients with CLL* increased percentages of lymphocytes bearing "B cell markers" was a characteristic feature. Data have been presented which might explain this on the basis of a monoclonal proliferation of the leukaemic lymphocytes from a B lymphocyte ancestor (section V:B:3). However, as seen in healthy donors, the various B cell markers are not expressed equally although in several patients all 3 markers were increased (see III, Table V). Some of these variations could be explained on the basis of varying amounts of normal T and non-T lymphocytes in these patients. Though, in CLL patients with high levels of peripheral blood lymphocytes, it is hard to see how the normal lymphocyte population could account for any major discrepancies among the B cell markers. Rather it seems as if there is a certain heterogeneity in the leukaemic lymphocytes in a single patient, in addition to the variations seen between different patients. As discussed above in connection with the normal donors, these variations might be explained by differences in the expression of individual markers during maturation of the lymphocytes. In accordance with this prediction Möller (1974) has found that the C3-receptor is lost fairly early during mitogen-induced transformation of mouse lymphocytes.

Studies of lymphocyte surface markers are of potential value for the classification of lymphoid cell malignancies although our picture of the expression of these markers on normal and leukaemic cells is far from complete. Further investigations, including kinetic studies, are needed to analyse the expression of these markers on individual cells. An explanation should also be sought why leukaemic lymphocytes in CLL generally arise from non-T cells, which only comprise a minor population of normal peripheral blood lymphocytes.

VI. HUMAN LYMPHOCYTE CYTOTOXICITY INDUCED BY TARGET CELL ANTIBODIES OR BY PHYTOHAEMAGGLUTININ

A. Basic Data

It is well established that the *in vivo* host versus graft reaction has an *in vitro* correlate in the cytolytic activity of the host's lymphocytes versus target cells from the

donor. This reaction is immunologically specific *i.e.* only target cells bearing the relevant transplantation antigens are destroyed. The specificity of the reaction has been attributed to committed T lymphocytes, acting as "killer cells" (for review see Cerottini and Brunner 1974).

In contrast to the specificity of this reaction non-immune lymphocytes activated by mitogens such as phytohaemagglutinin (PHA) show an indiscriminate cytotoxicity for a variety of target cells, even of autologous origin (Holm et al 1964, Perlmann et al 1968). Later, it became apparent that lymphocytes may be activated to cytotoxicity for suitable target cells in a variety of ways (for review see Perlmann and Holm 1969). For example, suitable target cells bearing small amounts of antibodies are lysed by purified peripheral blood lymphocytes from non-immune donors (see MacLennan 1972 a, Perlmann et al 1972 b). In this reaction the specificity of the lytic step is due to the humoral anti-target cell antibodies cooperating with non-immune lymphocyte effector cells.

In the sensitized target cell system strong cytolytic activity of the lymphocytes occurs even when extremely small amounts of antibodies (below 1 nanogram per 10^5 target cells) are present on the target cells (Perlmann et al 1972 a). However, whereas 7 S antibodies are efficient in mediating this effect, 19 S Ig is without sensitizing activity (MacLennan et al 1970, Perlmann and Perlmann 1970). Similar observations have been made in the Fc-rosetting reaction where 7 S but not 19 S antibodies are able to sensitize the indicator cells for opsonic adherence to human lymphocytes (III). It is thought that in the sensitized target cell system the cytotoxicity reaction is induced via a binding of the lymphocytic effector cells to immune complexes on the surface of the sensitized target cells. Accordingly, soluble immune complexes inhibit the cytotoxicity reaction (MacLennan 1972 b, Perlmann et al 1972 a). It is of interest that complexes formed between human IgG and human rheumatoid factors were efficient inhibitors of human lymphocyte cytotoxicity for target cells sensitized with rabbit antibodies (Hallberg 1972), indicating either that the receptor for rabbit IgG may bind human Ig or, alternatively, that there may exist Fc-receptors of different specificities on a single lymphocyte.

This latter study (Hallberg 1972) became the starting point for further investigations on the nature of the effector cells in antibody- and PHA-induced cytotoxicity, with particular emphasis on the role of IC-binding lymphocytes (IV, V). The results of these investigations are discussed below.

1. *Purity of effector cell populations in lymphocyte cytotoxicity experiments (IV, V)*

In the present investigations (IV, V) sensitized chicken erythrocytes were used as target cells, as originally described by Perlmann and Perlmann (1970). It is known that non-lymphocytic cells (granulocytes, monocytes) may destroy sensitized erythrocytes (for references see paper V) and therefore special precautions were taken to remove such phagocytes.

In paper IV lymphocytes were prepared by sedimentation of defibrinated blood in gelatine followed by nylon-wool filtration and centrifugation on a high-density

(Isopaque-Ficoll) solution leading to lymphocyte suspensions containing at most 0.7 % non-lymphocytic cells.

In paper V lymphocyte suspensions were prepared as described for the rosetting experiments (I, II, III), *i.e.* phagocytic cells were removed by the ingestion of iron particles followed by separation with a strong magnet. As an additional purification step differential centrifugation on a high-density solution was then performed. The purity of lymphocyte suspensions so prepared was 98 % or better. The advantage of this technique was that a possible separation of lymphocytes due to stickiness to nylon-wool was avoided. It is known that certain lymphocytes, preferentially non-T lymphocytes, may stick to glass and nylon (Wislöff and Fröland 1973).

The possibility must be realized that even when using purified lymphocyte suspensions a few remaining non-lymphocytic cells (granulocytes, monocytes), may exert cytotoxic activities against the target cells. However, morphological and kinetic studies have provided evidence that in the present systems the effector cells are indeed lymphocytes (for further discussion see paper V pp. 651–652 and Perlmann et al 1974).

B. Effect of Aggregated Human IgG on Antibody-Induced Cytotoxicity of Human Lymphocytes (IV)

Complexes between human IgG and human anti-immunoglobulin factors inhibit lymphocyte cytotoxicity for sensitized target cells (Hallberg 1972). This observation initiated studies to see whether polyclonal human IgG, not reacted in antigen-antibody complexes, would also inhibit lymphocyte cytotoxicity *in vitro*. In this investigation (IV) a commercial preparation of 99 % pure human IgG (gift of AB Kabi, Stockholm) was used. Solutions of this freeze-dried material were stored at -22°C and thawed only once, if not stated otherwise.

It was shown (IV) that this IgG preparation inhibited lymphocyte cytotoxicity in the sensitized target cell system and this inhibition was reversible, the cytotoxic activity being restored by an increase of target cell antibodies. Purification of this material by DEAE-cellulose chromatography or preparative agarose electrophoresis showed that the inhibitory activity could be ascribed to IgG (IV). Independently, Larsson et al (1973) and Wislöff et al (1974) have found human IgG to be inhibitory in antibody-induced lymphocyte cytotoxicity. That human IgG, when coated on to a suitable target cell may *induce* human lymphocytes to cytotoxicity was shown by Hayward (1974).

The inhibitory activity of various subclasses of human Ig has not been studied in the present investigation but experiments in Perlmann's and in MacLennan's laboratories have shown that the subclasses 1, 2 and 3 of human IgG are all inhibitory (MacLennan et al 1973, Larsson et al 1973). The capacity of IgG4 to inhibit lymphocyte cytotoxicity is still a matter of discussion, one group finding inhibitory activity (MacLennan et al 1973), others not (Larsson et al 1973, Wislöff et al 1974). In the interpretation of these results the possible differences in aggregation of various preparations must be included. Similar arguments may be raised to explain the differences in inhibitory activity seen between anodal and kathodal human IgG

TABLE 1. *Preincubation of human lymphocytes with human IgG at +4°C. Effect on the cytotoxic potential of the lymphocytes in antibody-induced lymphocyte cytotoxicity.*

Lymphocytes preincubated in	Per cent isotope release from ^{51}Cr -labelled, antibody-sensitized chicken red cells			
	Experiment 1		Experiment 2	
	8 h	16 h	8 h	16 h
Tissue culture medium only	25.8	50.5	49.6	63.4
Tissue culture medium containing				
HGG 5 mg/ml	7.5	24.5	31.0	53.1
HGG 0.5 mg/ml	ND	ND	43.3	63.0
HSA 5 mg/ml	29.5	43.5	50.2	64.2

15×10^6 lymphocytes in 3 ml of tissue culture medium were incubated at +4°C for 1 hour under gentle rocking. The lymphocytes were then washed 3 times at +4°C and used in a cytotoxicity assay against chicken red cells sensitized with a 5×10^{-5} dilution of anti-chicken red cell antiserum as described in paper IV. Means of duplicates in two independent experiments are shown. Spontaneous isotope release (2.2–2.4 per cent) has been subtracted.

ND = not done.

HGG = human IgG (AB Kabi, Stockholm).

HSA = human serum albumin (AB Kabi, Stockholm).

(IV). The inhibition of lymphocyte-mediated cytotoxicity for sensitized target cells by human IgG2 and possibly IgG4 stands in contrast to the cytolytic activity of human monocytes for antibody-coated erythrocytes, which is unaffected by IgG2 and IgG4 (Holm et al 1974).

The sensitizing antibodies on the chicken red cells required an intact Fc-fragment for inducing lymphocytes to cytotoxicity (IV, Table V). This is in accordance with the observations by Larsson and Perlmann (1972) and by Möller and Svehaug (1972). Furthermore, it was shown (IV) that pepsin digested human IgG, in which the Fc-fragment has been destroyed, no longer inhibited the cytotoxicity reaction. This indicated that the inhibition did not depend on the antigen-binding activity of the immunoglobulins. The possibility that the IgG might act on the target cells was excluded since extensive absorption of Ig solutions with chicken red cells did not reduce the inhibitory activity of human IgG in the cytotoxicity assay (Hallberg, unpublished data). Lymphocytes preincubated with human IgG at +4°C and then washed before use had a lower cytotoxic potential for sensitized target cells than had untreated lymphocytes (see Table 1). The inhibition was, however, less efficient than that seen when similar concentrations of human IgG were included in the cultivation medium.

In summary, several pieces of evidence indicate that polyclonal human IgG inhibits lymphocyte cytotoxicity for sensitized target cells by acting on the lymphocytes. The data are in accordance with the assumption that the inhibitory activity of IgG is due to a binding to Fc-receptors on the lymphocyte surface thereby blocking the attachment of the receptors to IC on the sensitized target cells. Studies by

MacLennan et al (1974) have indicated that the structure on IgG binding the first component of complement is distinct from that inducing lymphocyte cytotoxicity. They concluded that the heavy chain structures essential for combining with the lymphocyte are situated nearer the C-terminal end of the Fc part than are the receptor for C1q, and probably belong to the CH3 domain. In contrast Wislöff et al (1974) gave evidence that the region on the Ig molecule involved in binding to the Fc-receptor of the effector lymphocytic cell may be located within the CH2 domain. This point requires further study.

The finding that mixtures of human IgG and human anti-Ig sera were more inhibitory in antibody-induced lymphocyte cytotoxicity than either alone (Hallberg, 1972) anticipated that the degree of aggregation might be of importance for the inhibitory effect of IgG in the cytotoxicity assay. However, heat aggregated (63°C, 15 min) human IgG was, if anything, less inhibitory than unheated IgG (IV). Independently Larsson et al (1973) and MacLennan et al (1973) have reported that heating of human IgG at 63°C for 20–30 min had little effect on its inhibitory activity in antibody-induced lymphocyte cytotoxicity. These observations stand in contrast to the increased binding of complement and blocking of neutrophil phagocytosis by IgG after heat aggregation (MacLennan et al 1973).

Thus, experiments using heated human IgG did not indicate that aggregation was of importance for the inhibitory activity of Ig in antibody-induced lymphocyte cytotoxicity. However, heating is known to produce large aggregates of Ig and therefore a method producing preferentially intermediary complexes, that is repeated freezing and thawing (Hansson, 1968 a, b), was tried. Human IgG aggregated in this way was separated in the preparative ultracentrifuge and the fractions were then tested for inhibition of antibody-induced lymphocyte cytotoxicity (IV). It could be shown that the aggregated, high density material was then more inhibitory than was the low density fraction. Freeze aggregated IgG separated on Sephadex G-150 behaved in a similar way, aggregate-containing fractions being more inhibitory than 7 S material. Centrifugation in the analytical ultracentrifuge showed the aggregate-containing material to contain 9–10 S polymers of Ig.

It thus appears that small complexes of Ig are more efficient inhibitors of the cytotoxicity reaction than are monomeric IgG. In one experiment in paper IV monomeric 7 S IgG was completely devoid of inhibitory activity whereas in a second experiment (in which the 7 S fraction did contain 1 % aggregated material) some inhibition was seen. It is thought that polyclonal IgG inhibits the cytotoxicity reaction by binding to lymphocyte receptors for Ig. The binding strength would then increase if multiple binding sites were available, as is the case after aggregation of Ig. Other investigations for example those of Basten et al (1972 a) and Dickler and Kunkel (1972), indicate that lymphocytes inefficiently bind 7 S IgG, and generally normal human serum does not inhibit this cytotoxic system (Hallberg 1972 and others). It is still not known, however, if 9–12 S size complexes are optimal for the inhibition of antibody-induced lymphocyte cytotoxicity and it would be of interest to further analyze the inhibitory activity of a series of IgG aggregates of defined sizes. The difficulty in evaluating studies of this kind is the tendency of IgG to further aggregate during storage and handling. The finding

in the present investigation that the inhibition of IgG is predominantly due to small aggregates finds support in investigations of the inhibitory activity of immune complexes showing that this is predominantly due to small antigen-antibody complexes (MacLennan 1972 b). Also, inhibition of the Fc-rosetting reaction occurs in the presence of small antigen-antibody-complexes prepared in antigen excess (I).

It should be mentioned that Fröland et al (1974) have proposed that the binding of aggregated human IgG to human lymphocytes might not be equivalent to Ig-binding to lymphocyte Fc-receptors. In the present investigation (IV) it was shown that the inhibitory activity of IgG was lost after pepsin digestion and thus dependent upon an intact Fc-fragment. This does not mean, however, that the lymphocyte receptors for rabbit Ig, coating the target cells, and for human IgG, used as inhibitory substance, are necessarily identical. Further studies are needed to clarify whether several Fc-receptors of various specificities may occur on a single lymphocyte.

C. Effector Cells in Lymphocyte Cytotoxicity Induced by Target Cell Antibodies or by Phytohaemagglutinin (V)

1. Antibody-induced lymphocyte cytotoxicity

It was shown (Hallberg 1972, IV) that the antibody-dependent cytolytic reaction was inhibited by soluble immune complexes or by properly aggregated human IgG. It was proposed that this inhibition occurred via a binding of such complexes or aggregates to the effector lymphocytes leading to a competitive blocking of receptors on the lymphocyte membrane. However, alternative explanations are possible and to further analyze the cytolytic mechanism and the nature of the effector cells attempts were made to use the earlier described (I) Fc-rosetting reaction for a selective elimination of immune-complex-binding cells.

In these experiments (V) lymphocytes rosetted with sensitized ox RBC could be eliminated by centrifugation on a high density solution. The non-rosetted lymphocytes were then tested for cytotoxic activity. After exclusion of Fc-rosetting lymphocytes there was a considerable decrease in the cytotoxic activity of the lymphocytes in the sensitized target cell system (see Table 2). This finding lends support to the concept that such IC-binding lymphocytes have a central role in the lytic reaction and probably are the cells performing the lysis of the target cells. Attempts to isolate the IC-binding lymphocytes in a functionally active form have so far been unsuccessful. The final, formal proof, that these lymphocytes alone exert the cytotoxic activity for sensitized target cells thus remains. As discussed in papers I and III and in section V:A of this thesis Fc-binding, unstimulated lymphocytes are, with few exceptions, non-T cells. Evidence that non-T cells are effector cells in antibody-induced lymphocyte cytotoxicity has previously emerged from studies in animal systems (Harding et al 1971, van Boxel et al 1972, Britton et al 1973). However, data on whether these effector cells are Ig-bearing or not, have been more conflicting. Thus, Forman and Möller (1973), studying antibody-induced cytotoxicity by mouse spleen lymphocytes, concluded that the effector cell belonged to a subpopulation of B cells bearing surface Ig and Fc-receptors but incapable of

TABLE 2. *Lymphocyte cytotoxicity for sensitized chicken erythrocytes after exclusion of immune-complex-binding lymphocytes.*

Lymphocytes	Per cent isotope release in cytotoxicity assay (mean $\pm$ 1SD)	Per cent rosettes with sensitized ox RBC (mean $\pm$ 1SD)
Untreated (A)	71.9 $\pm$ 17.2	17.0 $\pm$ 8.0
Treated with un-sensitized ox RBC (B)	69.1 $\pm$ 18.7	22.6 $\pm$ 13.2
Treated with sensitized ox RBC (C)	8.6 $\pm$ 9.2	0.4 $\pm$ 0.9

Compiled data from 5 independent experiments in paper V. Comparisons of paired data in these 5 experiments are shown below.

Differences in per cent isotope release

A-B: Mean 2.80; SD= 3.90; t= 1.60; DF= 4; P>0.05
B-C: Mean 60.56; SD= 11.72; t= 11.55; DF= 4; P<0.001.

differentiating into antibody-secreting cells. In contrast, Greenberg et al (1973) proposed that antibody-dependent cell mediated cytotoxicity expressed by mouse spleen lymphocytes was due to a "null" lymphoid cell not bearing surface Ig or T cell determinants. Rech et al (1974) gave evidence that in the rabbit Ig-bearing lymphocytes may function as effector cells for antibody-sensitized target cells. Perlmann et al (1972 b) have separated human lymphocytes on Ig-anti-Ig columns and shown retention of effector lymphocytes, but it was not unequivocally established whether this was due to the anti-Ig activity or to the presence of IC in these columns. Evidence against the former interpretation was presented by Wislöf and Fröland (1973), who excluded Ig-bearing peripheral blood lymphocytes by filtration through nylon-wool columns without getting reduction in cytotoxic activity. Similar results were obtained by Perlmann et al (1974) who showed that effector lymphocytes pass through columns charged with pepsin fragments of anti-Ig antibodies, which retain Ig-bearing lymphocytes.

It must be stressed that it is not established that *all* Fc-binding lymphocytes are capable of acting as effector cells for sensitized target cells. Kedar et al (1974) argue that only a subpopulation of Fc-binding lymphoid cells in the rat has this capacity. Also, in addition to the Fc-receptor, the effector cells in the human seem to carry receptors for C3b since filtration of lymphocyte suspensions on C3b-loaded columns abolished the activity of the lymphocytes (van Boxel et al 1973, Perlmann et al 1973 a, b).

2. *Phytohaemagglutinin-induced lymphocyte cytotoxicity*

From a mechanistic point of view it was expected that the effector cells in antibody-mediated lymphocyte cytotoxicity would carry receptors for IC. More unexpectedly

TABLE 3. *Phytohaemagglutinin-induced lymphocyte cytotoxicity for chicken erythrocytes after exclusion of immune-complex-binding lymphocytes.*

Lymphocytes	Per cent isotope release in cytotoxicity assay (mean $\pm$ 1SD)	Per cent rosettes with sensitized ox RBC (mean $\pm$ 1SD)
Untreated (A)	49.2 $\pm$ 10.4	15.1 $\pm$ 6.7
Treated with unsensitized ox RBC (B)	40.8 $\pm$ 13.0	19.9 $\pm$ 10.7
Treated with sensitized ox RBC (C)	19.1 $\pm$ 11.3	0.2 $\pm$ 0.7

Compiled data from 9 independent experiments in paper V. Comparisons of paired data in these 9 experiments are shown below.

Differences in per cent isotope release

A-B: Mean 8.40; SD=11.55; $t=2.18$; DF=8; $P>0.05$

B-C: Mean 21.63; SD=10.97; $t=5.90$; DF=8; $P<0.001$.

Differences in per cent rosettes with sensitized ox RBC

A-B: Mean -4.78; SD= 4.05; $t=3.53$; DF=8; $P<0.01$

B-C: Mean 19.66; SD=10.30; $t=5.72$; DF=8; $P<0.001$.

it was found that lymphocyte cytotoxicity in the PHA system was also inhibited by removal of IC-binding lymphocytes by the Fc-rosetting technique (V). This inhibition was less pronounced than that seen in the sensitized target cell system but was highly significant and averaged approximately 50 % of the mean cytotoxic activity of lymphocytes treated with unsensitized ox RBC (see Table 3). This finding suggests a role for IC-binding lymphocytes in PHA-induced cytotoxicity and stands in contrast to earlier observations that soluble IC do not inhibit PHA-induced lymphocyte cytotoxicity (Hallberg 1972, Perlmann et al 1972 a, b). However, in the present system high concentrations of sensitized ox RBC (but not unsensitized ox RBC) indeed reduced the cytotoxicity in the PHA system (Table 4).

In the separation experiments (V) lymphocyte suspensions treated with unsensitized ox RBC had a slightly higher percentage of Fc-rosettes compared to untreated lymphocytes. There are now several reports showing that red cells other than sheep RBC may bind to human lymphocytes (Stathopolous and Elliot, 1974 and others). One possible explanation would then be that in the present investigation (V) some lymphocytes were trapped among the non-sensitized ox RBC during the separation procedure, although true rosettes were never seen. It should be emphasized, however, that no significant difference was seen between untreated lymphocytes and lymphocytes treated with unsensitized ox RBC when the cytolytic activity of the lymphocytes was tested in antibody-mediated or PHA-induced cytotoxicity (see Tables 2 and 3).

Earlier investigations have stressed the role of T lymphocytes in PHA-induced lymphocyte activities (see Transplant. Rev. 1972, vol. 11). However, PHA does

TABLE 4. *PHA-induced lymphocyte cytotoxicity for chicken erythrocytes. Effect of admixture of sensitized or unsensitized ox red cells.*

Admixture of ox red cells to lymphocytes	Per cent isotope release from ⁵¹ Cr-labelled chicken red cells	
	Medium + PHA	Medium
None	47.9	6.4
Unsensitized ox RBC		
5 × 10 ⁴	48.2	4.6
5 × 10 ⁵	46.3	2.8
5 × 10 ⁶	44.5	2.1
Sensitized ox RBC		
5 × 10 ⁴	38.8	7.4
5 × 10 ⁵	33.6	4.1
5 × 10 ⁶	25.3	2.3

The cytotoxic activity of 5 × 10⁵ lymphocytes against 10⁵ chicken erythrocytes was tested in the presence of various numbers of ox red cells sensitized with rabbit antibodies (Fc-receptor indicator cells). Ox red cells treated with normal rabbit serum were used as control (unsensitized ox RBC). Incubation time 40 hours. Final dilution of PHA 1:1000. Experimental conditions as in paper V.

bind to mouse B lymphocytes (Greaves et al, 1972, Stobo et al 1972) and insolubilized PHA activates mouse B lymphocytes to transformation (Greaves and Bauminger, 1972). It is known that the formation of "complexes" between PHA and red cell membranes may facilitate the mitogenic activity of PHA for lymphocytes (Tärnvik 1970, 1971). Possibly this effect is related to the insolubilization of PHA on the red cell membrane. It is conceivable that in the present cytolytic system, utilizing chicken red cells as the target cells, part of the PHA is bound to the membranes of the red cells in such a way as to function as a non-soluble mitogen capable of activating non-T lymphocytes as well as T lymphocytes.

Direct evidence that non-T cells are participating in PHA-induced lymphocyte cytotoxicity has been presented by Britton et al (1973) showing inhibition of this reaction in the mouse by a mouse anti-B lymphocyte antiserum. In this context it is interesting that Schiff et al (1974) found impaired transformation in the presence of PHA of lymphocytes from patients with X-linked agammaglobulinemia again indicating that lymphocytes other than T lymphocytes may be activated by PHA.

In summary then, it is well established that PHA-induced cytotoxicity is dependent upon a T cell population, but several data, including those presented in paper V, indicate that a non-T cell population may participate as well, at least under certain experimental conditions. Thus, there might be a co-operation between several lymphocyte populations in the cytotoxicity process induced by PHA, in analogy to the T-B co-operation in humoral immune responses to thymus dependent antigens. Such a co-operation might explain the longer induction period in the PHA system compared to antibody-dependent lymphocyte cytotoxicity (32-40 hours versus 4-8 hours). It may be speculated that different cell populations are involved in the induction and the effectuation of PHA-induced lymphocyte cyto-

toxicity. Alternatively, PHA may activate two (or more) independent cell populations, each capable of completing the cytotoxicity reaction. Studies of seemingly artificial mitogens such as PHA and concanavalin A have brought new insights in the functions of lymphocyte subpopulations (Transplant. Rev. 1972, vol. 11). The mitogen-induced lymphocyte cytotoxicity system may be equally suitable for elucidating certain aspects on the complex interrelations among various lymphocyte subpopulations.

VII. SUMMARY

A. Methodology

1. For the demonstration of binding of immune complexes to human lymphocytes a method was devised (I) of a rosetting reaction using as the indicator cells ox RBC sensitized with rabbit antibodies. Rabbit 7S but not 19S antibodies were efficient for mediating this reaction (III), and the reactivity was expressed by live lymphocytes only.
2. The mixed antiglobulin reaction for detection of Ig-bearing lymphocytes was modified by pretreating the lymphocytes in a formaldehyde fixative (II). This procedure provided several advantages over the earlier used low temperature storage technique:
 - a) Higher percentages of reacting lymphocytes, well on a level with those found by other investigators using immunofluorescence techniques.
 - b) Independence, within a wide range, of concentration of the indicator red cell suspension, in contrast to the earlier method.
 - c) Possibility to store lymphocytes for several days before testing, with retained consistency in the results.
 - d) Inability of formaldehyde-treated lymphocytes to form rosettes in other test systems (e.g. Fc-rosettes, C3-rosettes, sheep RBC-rosettes), thereby efficiently reducing the occurrence of non-specific phenomena in the mixed antiglobulin reaction.
3. The Fc-rosetting procedure was applied for fractionation of lymphocytes to be tested in cytotoxicity assays (V). By centrifugation on a high density solution rosetted lymphocytes could be removed from the suspension. The remaining cell population, lacking Fc-binding lymphocytes, was used in cytotoxicity experiments for a study of effector cell populations in antibody-induced and PHA-induced lymphocyte cytotoxicity (V).

B. Studies on Human Lymphocyte Surface Markers and Human Lymphocyte Cytotoxicity in Vitro

It may be relevant here to repeat the questions forwarded in "AIM OF THE INVESTIGATION" and to give the answers found.

1. *How are 4 cell surface markers (i.e. receptors for immunoglobulin, receptors for complement, surface immunoglobulin determinants and receptors for sheep red cells) distributed among peripheral blood lymphocytes in healthy humans as compared to patients with chronic lymphatic leukaemia? Do individual lymphocytes bear more than one of these markers?*

In papers I and II CLL patients were shown to display increased percentages of Fc-binding and Ig-bearing peripheral blood lymphocytes compared to normal donors. In paper III all 4 different rosetting reactions mentioned above were applied to the lymphocytes of 20 healthy donors and 11 CLL patients. In the CLL group increased values were seen in the mixed antiglobulin reaction for detection of surface Ig determinants, as well as in the Fc-rosetting test and C3-rosetting test. Concomitantly, there was a decrease in sheep RBC-rosettes in these patients. These results were consistent with the notion that sheep RBC rosettes mark a cell population, probably T cells, different from those marked by surface Ig, Fc-rosettes and C3-rosettes. Also a minor population of lymphocytes showed receptors for sheep RBC and either of Fc or C3 as tested in a mixed rosetting technique on lymphocytes from normal donors.

2. *Does polyclonal human IgG inhibit the in vitro cytotoxicity of non-immune human lymphocytes for target cells coated with rabbit antibodies? If so, is the inhibitory activity of IgG dependent upon IgG aggregates, interacting with the effector lymphocytes?*

In paper IV such an inhibitory activity of polyclonal human IgG was shown to occur and the inhibition was dependent upon an intact Fc-fragment of the Ig. Small Ig complexes of 9 to 10S were more inhibitory than were monomeric molecules or heavy weight aggregates. It was proposed that this inhibition was due to the binding of Ig aggregates to Fc-receptors on the cytotoxic lymphocytes.

3. *Are lymphocytes bearing receptors for immunoglobulin needed in lymphocyte cytotoxicity reactions induced by target cell antibodies or by phytohaemagglutinin?*

In paper V it was shown that after exclusion of IC-binding lymphocytes human lymphocyte cytotoxicity for antibody-coated target cells was profoundly reduced. Lymphocyte cytotoxicity induced by PHA was also reduced by the same procedure. It was concluded that a population of IC-binding lymphocytes is needed for lymphocyte cytotoxicity induced by target cell antibodies and for the full expression of PHA-induced cytotoxicity.

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